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Characterisation and functionalisation of protein from brewer's spent grain and its application in novel beverages

Thesis presented by

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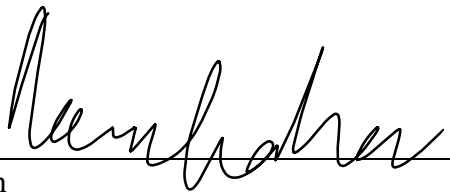
Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.



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Abbreviations

ABP	Animal based protein
AJ	Apple juice
BCAA	Branch chain amino acids
BPI	Beef protein isolate
BRP/BRPI	Barley and rice protein isolate
BSG	Brewers spent grain
BT	Black tea (unsweetened)
CP/H	Collagen protein/hydrolysate
DIASS	Digestible indispensable amino acid score
EAA	Essential amino acids
EDF	EverPro Dark Fraction
ELF	EverPro Light Fraction
EP	EverPro
IT	Ice tea (sweetened)
L*	Lightness
L. amylovorus FST 2.11	*Lactobacillus amylovorus* FST2.11
L. citreum TR116	*Leuconostoc citreum* TR116
L. plantarum FST1.7	*Lactiplantibacillus plantarum* FST1.7
LAB	Lactic acid bacteria
LL	Lemon and lime
LL Free	Lemon and lime sugar free
LOD	Limit of detection
MPC/I	Milk protein concentrate/isolate
MRS	DeMan Rogosa and Sharpe
N	Nitrogen
NAB	Non-alcoholic beer
PBP	Plant-based protein
PCA	Principal compoenent analysis
PPI/H	Pea protein/isolate/hydrolysate
RP	Rice protein isolate
RTD	Ready to drink
SD	Soft drink
SPI	Soy protein isolate
TTA	Total titratable acidiy
WPC/I/H	Whey protein concentrate/isolate/hydrolysate

Abstract

Barley and rice protein isolate (BRPI), upcycled from brewers spent grain (BSG), is a major byproduct of the brewing industry. This thesis investigated the characterisation and functionalisation of BRPI with a focus on its application in novel beverages, specifically acidic, water-based beverages where incorporation of plant-based proteins (PBP) faces solubility, stability and sensory challenges. In particular, this research explores BRPIs composition, techno-functional properties, sensory characteristics and its utilisation in acidic water-based, soft drinks. Initial stages involved a comprehensive retail market survey and physicochemical analysis of animal and plant derived protein soft drinks, identifying gaps in formulation and assessing the scarcity of PBP soft drink options. Among the 138 protein soft drinks surveyed, animal-based proteins (ABP) dominated the market, primarily whey protein isolate (WPI) and collagen hydrolysate (CH). Only 18% of the products contained PBP options, predominantly formulated with pea protein isolate/hydrolysate (PPI/H) or single amino acids. The digestibility characteristics of BRPI, determined using the dynamic tiny-TIM *in vitro* model, achieved a nitrogen bio-accessibility >90% and a DIAAS of 67% (lysine-limited) that outperformed conventional PBP isolates including pea, soy and rice while closely matching WPI's techno-functional properties. Decolourised BRPI, lighter in colour than original BRPI, were both characterised and compared to assess differences in functionality and sensory quality. Both exhibited high solubility (>80%) across various pH ranges, including acidic conditions. Decolourised BRPI displayed higher lubricating properties and reduced flavour profiles due to reduced Maillard reaction associated aroma compounds. Application trials were conducted across a range of commercially available soft drink and non-alcoholic beverage systems to evaluate the impact of BRPI, at various concentrations, on their functional properties. The protein concentration added affected the pH, stability and viscosity of the formulations. Fermentation of BRPI with three different lactic acid bacteria (LAB) (*Lactiplantibacillus plantarum* FST 1.7, *Leuconostoc citreum* TR116 and *Lactobacillus amylovorus* FST 2.11) demonstrated its suitability as a substrate for producing high protein fermented beverages. The strains showed specific differences in their techno-functionality and sensory attributes including fruity and dairy like flavours. While all strains showed acidification only TR116 produced mannitol. This research provides approaches for valorising brewing side streams for novel high protein beverages that can help support consumers struggling to meet adequate protein requirements.

1. Chapter 1

Introduction

Global demand for protein is increasing rapidly, driven by a projected population of 10 billion people by 2050, alongside socio-economic changes with rising incomes and greater consumer awareness of the role of protein in health and healthy ageing (Henchion et al., 2017; United Nations, 2022). As a result, the need to diversify protein sources beyond conventional animal-based (ABP) options has intensified, with plant derived sources receiving attention with their potential to address environmental and ethical concerns.

A wide range of plant-based protein (PBP) sources are currently available, including legumes such as soy, pea, chickpea and cereals including oat, wheat and rice with emerging sources from faba bean, lentil and quinoa (Henchion et al., 2017; Hoehnel et al., 2022). However, the successful incorporation of these ingredients in food and beverage systems depends largely on their techno-functional properties. Food and beverage manufacturers typically prioritise ingredients at a competitive cost with desirable functional characteristics, including protein solubility, gelation, emulsification and foaming, all of which determine their suitability for specific product applications.

Despite their sustainability advantages over ABP, many PBPs are derived from crops cultivated specifically for protein extraction. This process can generate substantial amounts of side streams such as starch and fibre rich fractions, that remain underutilised in human nutrition. Improving the valorisation of existing food and beverage processing side streams can address this issue to maximise resource efficiency. Several such side streams explored for the recovery of protein isolates/hydrolysates include soy, corn distillers dried grain, canola and corn meal as well as barley brewers spent grain (Eastham et al., 2023).

Among these, brewers spent grain (BSG), is produced in particularly large quantities by the brewing industry. In the brewing process, raw barley and malted barley are the most commonly used grains often in combination with adjuncts like rice for lager type beers. Brewers spent grain is the remains of the initial brewing grains after being subjected to the malting and mashing processes, accounting for approximately 85% of total brewery side streams. It is generated at an estimated rate of 20 kg wet BSG per 100 L of beer produced (Jaeger et al., 2021; Mussatto et al., 2006). Traditionally, BSG has been used for animal feed or discarded as

waste. However, its rich composition of protein (approximately 19 – 30%), predominantly hordeins and glutelins as well as carbohydrates and fibre, suggests extracting the protein can therefore provide a valuable source of plant protein for food and beverage formulations (Jaeger et al., 2021).

Protein from dried BSG can be extracted using a variety of methods, with alkaline extraction being the most common approach, often in combined with enzyme treatments using carbohydrases and proteases for improved yield (up to 80%). The solubilised proteins are then recovered through centrifugation, isoelectric precipitation and/or membrane filtration (Devnani et al., 2023). Industrial approaches to valorise BSG is already emerging. The beer company Anheuser-Busch InBev created a spin off company, EverGrain, who upcycle their BSG from a malted barley and rice adjunct brew. Proteins are extracted using a series of enzymatic hydrolysis, sized based separation and purification steps to produce a barley and rice protein isolate (BRPI) called EverPro (Turck et al., 2023). Two variants are available, EverPro Dark Fraction (EDF) and EverPro Light Fraction (ELF), both containing a mixture of barley and rice proteins. These ingredients are commercially available largely in the United States (US) and Europe (EU).

Upcycling of BSG into value added protein ingredients aligns with the principles of a circular economy and contributes directly to the United Nations Sustainable Development Goals (SDG's), particularly SDG 2 (zero hunger), SDG 3 (good health and wellbeing), SDG 9 (industry, innovation and infrastructure), SDG 12 (responsible consumption and production) and SDG 13 (climate action) by providing high protein ingredients through by product valorisation, reducing waste (McTernan et al., 2025). Alongside ethical and environmental motivations, the exploration of underutilised by-products such as protein from BSG, BRPI, provides a route to expand the PBPs available to industry while improving resource efficiency.

The incorporation of PBPs into dairy alternative beverages such as plant-based milks, plant protein shakes and ready to drink (RTD) meal replacers is already well established (Irondi et al., 2025; Jeske et al., 2017; Mäkinen et al., 2016), while their application in other beverage categories remains limited. At the same time, the functional beverage market is one of the fastest growing sectors of the global beverage industry. These products are formulated with ingredients such as vitamins, minerals, herbs, caffeine, fibre and/or protein, to provide benefits beyond hydration including enhanced energy, performance or gut health. Common examples include energy, sports and fermented drinks (Gupta et al., 2023). Within this context, soft

drinks can be defined as non-alcoholic, water-based beverages formulated with flavourings, sweeteners, and acidulants, which may be carbonated or non-carbonated, characterised by an acidic environment (Ashurst et al., 2017).

In these beverages, protein fortification is currently dominated by ABPs, particularly whey and collagen. These proteins are widely used due to their high solubility over a range of pH values, favourable functional and sensory properties, in addition to their high nutritional quality (Liu et al., 2020; Prado et al., 2015; Singh et al., 2014; Yadav et al., 2016; Zhang et al., 2023). However, interest in incorporating PBPs into these systems is increasing. The acidic water-based characteristics of these beverages presents an untapped opportunity for the incorporation of PBPs to create hydrating protein enriched soft drinks.

The development of protein soft drinks is increasingly driven by consumer nutritional demands particularly for convenient RTD products that support adequate protein intake. Such products can offer benefits to a wide range of population groups. Athletes, for example, require higher protein intakes, approximately 1.4 to 2g of protein per kg of bodyweight per day, for building and maintaining muscle mass (Jäger et al., 2017). Beyond, these sports enthusiasts, the growing use of glucagon like peptide-1 (GLP-1) therapies for weight management in overweight and obese individuals has created new nutritional challenges. While these drugs effectively supress appetite and promote weight loss, they can inadvertently reduce overall food intake making it difficult to meet adequate protein needs, increasing the risk of muscle loss or sarcopenic obesity (Fitch et al., 2025). Additionally, individuals following plant-based, vegetarian or stricter vegan diets may struggle to achieve adequate protein intake. Ready to drink protein-fortified beverages incorporating PBP can serve as an effective way to help these diverse groups meet their protein requirements.

However, incorporating plant isolates/hydrolysates into such water based, acidic beverages present significant technical challenges. These include poor solubility, protein precipitation and off flavours, all of which negatively impact product stability and sensory quality (Bessada et al., 2019; Gorissen et al., 2018; Ma et al., 2022). These limitations have contributed to the slower application of PBP in soft drinks compared with ABPs. It has been reported that protein from BSG demonstrates high solubility at neutral pH compared to other PBPs studied, but their behaviour in acidic water-based systems remains largely unexplored (Jaeger et al., 2023; Tang et al., 2023).

Collectively, these findings highlighted a gap in the current literature and a clear opportunity for further exploration. Therefore, the aims of the thesis were to evaluate the nutritional, techno functional and sensory properties of BRPI in novel beverage systems, specifically acidic water-based beverages such as soft drinks. By addressing both industry needs and consumer demands, this work provides critical scientific and practical insights to support the commercial application of BRPI in developing hydrating high protein beverages, bridging the gap between waste valorisation, alternative PBP sources and beverage innovation.

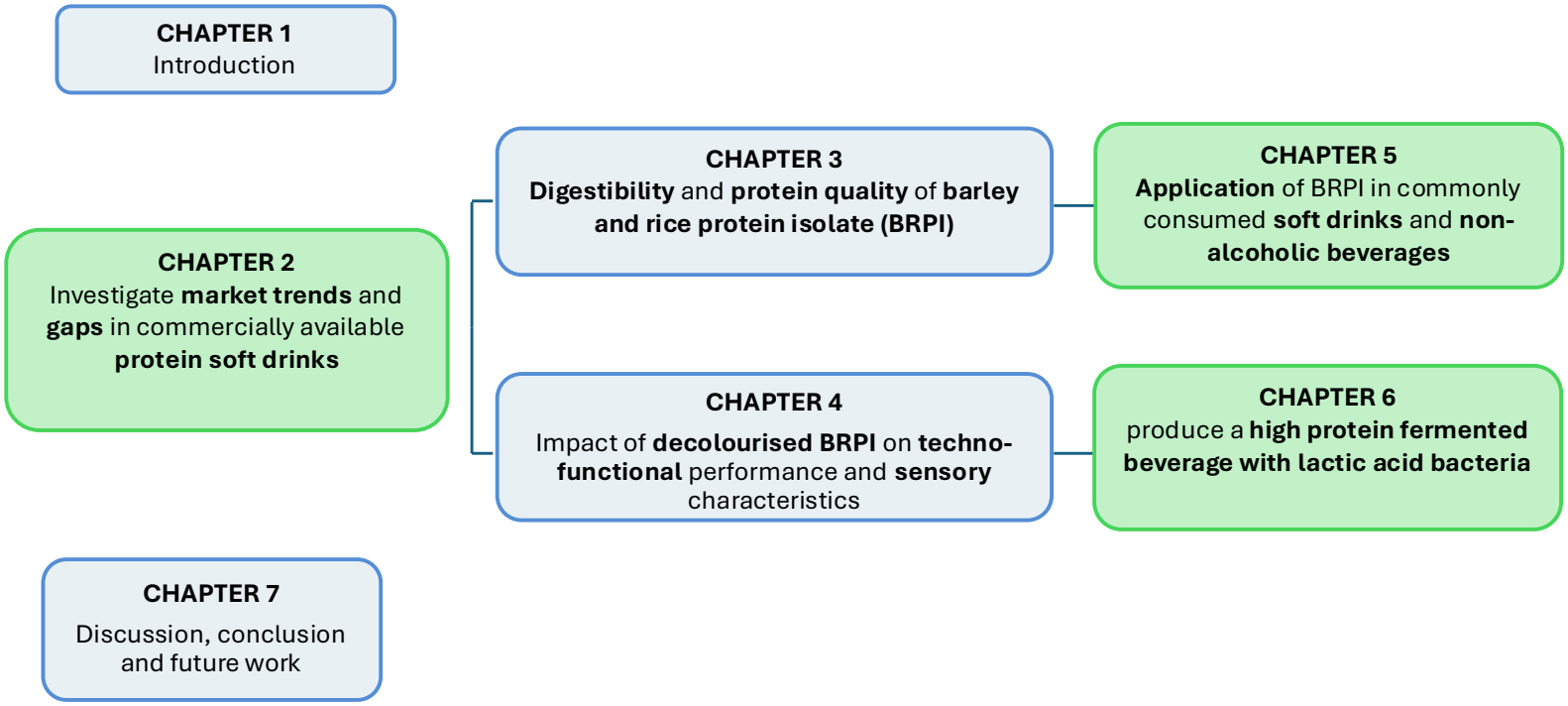
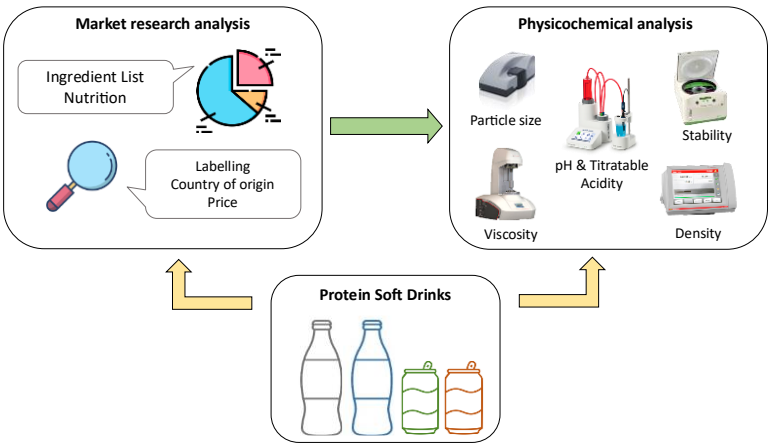


Figure 1.1: Process flow of thesis.

2. Chapter 2

Protein Soft Drinks: A Market Trends Analysis and Selected Product Characterisation



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Abstract

The market for protein-based drinks is endlessly growing, as the awareness of health-conscious consumers demands a shift from traditional protein smoothies or shakes to clear beverage alternatives that address thirst and hydration. The aim of this study was to investigate the soft drink market on a global scale with a focus on commercially available high protein soft drinks, carbonated and uncarbonated from both animal and plant-based protein sources. Additionally, the physicochemical properties of 25 selected protein soft drinks from the market research were evaluated including their protein content, density, viscosity, particle size, stability, pH and total titratable acidity (TTA), to explore their quality attributes. From the market research, 6.8% was the highest protein content found out of 138 beverages, with whey protein isolate (WPI) and collagen hydrolysate (CH) being the most popular added protein ingredients. Only 18% of the market contained plant-based proteins with pea protein isolate (PPI) being the most common. The pH of all beverages showed acidic values (2.9 to 4.2), where TTA ranged from 0.4 to 1.47 ml (0.1 M NaOH/ml). Protein content, density and viscosity in all beverages exhibited a significantly strong positive correlation. The protein soft drink containing beef protein isolate (BPI) stood out regarding highest protein content, density, particle size and TTA. Overall, these results demonstrate the effects and correlations of the different formulations on the quality characteristics. Therefore, the presented results can be utilised in the development and formulation of future protein soft drinks, including nutritional improvement and optimum quality, meeting current consumer trends, that is used as a convenient pre or post workout drink for individuals seeking muscle growth and repair.

2.1. Introduction

Soft drinks continue to be a popular choice of beverage where a recent survey by the EU commission in 2019 showed that 19% of individuals over the age of 15 said that they consumed sugar sweetened soft drinks up to 1 – 3 times a week where 9% of these individuals reported they consumed these types of beverages at least once a day (EU Commission, 2019). Soft drink trends began with beverages containing simple formulations mainly composed of sugar, water and a flavouring, often carbonated, obtaining little to no nutritional value. Due to health-conscious consumers these trends changed to sugar reduction, attributed from the implementation of the sugar taxes which lead to low-calorie and diet soft drinks (Popkin & Hawkes, 2016). This was done through reformulation, by replacing the sugar with non-calorific sweeteners. This was followed by the fortification of these beverages with additional ingredients such as botanicals, vitamins, minerals as well as added caffeine. These ready to drink (RTD) beverages can be either carbonated or non-carbonated (Ashurst, 2016).

Unless intentionally added, soft drinks contain a negligible amount of protein (Ashurst, 2016). Driven by the rising awareness of health-conscious consumers, the market for protein-based beverages continues to expand. These consumers are seeking a shift away from traditional protein smoothies or shakes that can be associated with heavier consumption due to their thick texture, to lighter clear beverage options that can address thirst and hydration. Beverages such as these can be a convenient option to supply a high dose of protein. Multiple population groups may benefit from these products, including athletes who require higher protein intakes to support muscle maintenance and growth but also for individuals using glucagon 1 peptide (GLP-1) therapies who are at risk of muscle loss due to reduced food intake and consequently inadequate protein consumption (Fitch et al., 2025; Jäger et al., 2017). Additionally, consumers following plant-based like vegetarian and vegan diets, for additional options to help meet their protein needs.

The functional drinks market is one of the fastest growing industries, with one of the most popular functional beverages on the market being functional waters (such as vitamin and mineral fortified drinks, sports and energy drinks, herbal drinks and health and wellness drinks) (Gupta et al., 2023). The global functional beverage market is estimated to reach $153 billion US dollars in 2023 and expand to $279 billion US dollars in 2030 (Gupta et al., 2023). Recently, the fortification of these types of beverages with protein such as soluble whey is emerging (Goudarzi et al., 2015; Ashurst, 2016; Prado et al., 2015). Beauty drinks have also started to

increase in popularity such as in fruit juices with collagen peptides to improve skin hydration which are trendy in the Asian market and increasing in the US market (Polonini et al., 2021). Additionally, more consumers are shifting towards a more ethical diet and as a result, the food industry is developing an increasing number of plant-based products (Arbach et al., 2021). Many studies are focused on milk alternatives and their quality improvement, and little research has been shown on alternative protein enrichment of flavoured beverages as well as the nutritional improvement on beverages such as soft drinks (Jeske et al., 2017; Vogelsang-O'Dwyer et al., 2021). Protein isolates are becoming widely suitable for the use in acidic drinks (Goudarzi et al., 2015; Prado et al., 2015). Furthermore, incorporating more plant proteins in western diets are among the current and future global trends for the food and beverage industry (Mintel, 2023).There has been limited research shown regarding a detailed retail market analysis of protein-enriched soft drinks, along with functional properties associated with these products. Therefore, the aim of this study was to, firstly, investigate the global market for commercial high protein soft drinks, carbonated and uncarbonated. Therefore, 138 selected protein beverages were systematically evaluated regarding their nutritional dimension (product description, list of ingredients, nutritional value, protein source) and economic dimension (country of origin, price, labels/claims). The second part of this study includes the analysis of the physicochemical properties of 25 selected protein soft drinks including eleven different brands with various flavours. Both animal and plant-based protein sources were assessed in this study.

2.2. Materials and methods

2.2.1. Part 1: Retail Market analysis

The global soft drink market, (Austria, Australia, Brazil, Canada, Germany, India, Portugal, Malta, New Zealand, South Korea, Sweden, UK and the USA), was investigated by accessing product availability on various sport, fitness and nutrition related online retailers, as well as commercial food and beverage websites (see **Supplementary Table 2.1**). The search for soft drinks was focused on water-based flavoured drinks and therefore, juices and smoothies were excluded from this study. A variety of high protein soft drinks (carbonated and uncarbonated) were collected including a variety of animal and plant-based protein (PBP) sources. One-hundred-and-thirty-eight protein soft drinks of 46 different brands with various flavours were found, of which included 35 carbonated and 103 uncarbonated. These products were analysed regarding their product descriptions (from the retailer website), list of ingredients (protein type, sweeteners, acidulants, preservatives, emulsifiers, stabilizers, antioxidant and antifoaming agents), nutritional value (protein content, amino acid profile, calorie content), labelling on the package (allergens, 'free-from', nutritional labels, 'zero', 'no added'), price (converted to € per 100 ml) and the country of origin. This market study was carried out from April 2022 until January 2023.

2.2.2. Part 2: Protein content and physicochemical analyses of selected protein soft drinks

Twenty-five commercial high protein soft drinks from the market research study were selected based on their regional availability, purchased, and analysed regarding their protein content and physicochemical characteristics. Amongst the 25 different soft drinks, 18 included animal-based protein, (8 different brands) and 7 included plant-based protein, (3 different brands) with various flavours. The selected soft drinks (SD) were numbered from 1-11 referring to the product brand and the corresponding letter (a – d) refers to different flavours of the same brand. An asterisk (*) refers to if the beverage was carbonated. The beverages were stored at 4°C and used within a day of opening. All analysis were performed on uncarbonated beverages. The carbonated beverages were decarbonated using an ultra-sonification bath (USC500TH, Hilton instruments, UK) at 20°C for 30 mins at a frequency of 45 kHz.

2.2.2.1. Protein determination

The protein content was measured on each beverage using the Kjeldahl method AACC Method 46-12 (AACC International, 2011). The total nitrogen content was determined and reported as well as total protein percentage was calculated based on a nitrogen to protein conversion factor of 6.25.

2.2.2.2. pH and Total Titratable Acidity

The pH of each commercial protein soft drink was measured using a pH meter (Mettler Toledo, Ohio, United States) at 20°C. Total titratable acidity (TTA) was determined using the method from Marchan et al. (2021), with modifications. An automated titrator (Easyplus, Mettler Toledo, Ohio, United States) was used for the addition of 0.1 M NaOH to 10 ml until a pH value of 7 was reached. The amount of 0.1 M NaOH used to reach pH 7 was divided by 10 to calculate the total titratable acidity per 1 ml of sample.

2.2.2.3. Particle Size Distribution

Particle size (nm) and polydispersity index (PDI) of each soft drink was measured using dynamic light scattering technology with the Zetasizer Nano Z (Malvern Instruments Ltd., Worcestershire, United Kingdom), equipped with a 633 nm laser. A refractive index of 1.45 and absorbance at 0.001 for proteins was used at 25°C. A PDI value of < 0.05 indicates a monodisperse samples whereas values > 0.7 are considered to obtain a broader size distribution of particles or regarded as polydisperse (Mudalige et al., 2018).

2.2.2.4. Density

The density (g/cm^3) of the soft drinks was determined using the Anton Paar density meter DMA 4500 M (Anton Paar GmbH, Austria) as described by Nyhan et al. (2023). Samples were centrifuged at 4893 x *g* for 5 min to remove any insoluble solids. The density of the supernatant was then measured at 20°C.

2.2.2.5. Apparent Viscosity

Rheological behaviour was performed as described by Jeske et al. (2017) with modifications. The apparent viscosity of the beverages was determined using a rheometer (MCR301, Anton Paar GmbH, Austria) equipped with a concentric cylinder measuring attachment (Anton Paar, GmbH, Austria). The dynamic viscosity (mPa·s) was measured as a function of shear rate ranging from 0.5 to 100 (1/s) at 6°C. Then the apparent viscosity at 80.7 (1/s) was evaluated and used to compare the beverages with one another.

2.2.2.6. Phase Separation

Stability was determined using a Lumisizer (LUM GmbH, Berlin, Germany), through phase separation based on light transmission during centrifugation. The separation rate, an indication of stability, was calculated by the software. The measurement involved two cycles at 5 x *g* for 2 min and 1878 x *g* for 50 min, at a wavelength of 865 nm and temperature of 25°C. During centrifugation, the samples were illuminated with near infrared light, and transmitted light was detected by sensors across the entire sample. The percentage of integrated light transmission increased over time which is then calculated by the software as described by Vogelsang-O'Dwyer et al. (2021) and the separation rate in % transmission is given per minute.

2.2.2.7. Colour

The CIE L* a* b* and RGB measurements of the samples were completed with a UV-VIS spectrophotometer (Thermo Scientific Genesys 50, Waltham, MA, USA) using the method by Delgado-González et al. (2018) calculated according to CIELab parameters at illuminant D65. Briefly the colour coordinates of the beverages were determined by transmittance spectrum of the sample in the range 380 - 780 nm. The spectrum was recorded with a wavelength resolution in 5 nm steps between each measurement and blanked with distilled water. The colour index for L* represents black to white (0 to 100), a* ranges from green to red (-120 to 120) and b* from blue to yellow (-120 to 120).

2.2.2.8. Statistics

All analyses were performed in triplicate. A one-way ANOVA with post hoc Tukey test ($p < 0.05$) was performed using IBM SPSS version 26 (Armonk, NY, USA). The level of significant differences within the analysis performed was defined between all samples. Principal component analysis was performed using the software Origin lab pro 2023b (MA, USA), where the regression (*p*-values) and correlation (*r*-values) analysis was performed using Microsoft Excel 2021.

2.3. Results

The current study includes two parts: Part 1 reveals the market situation of 138 protein soft drinks, including beverage description, nutritional value and economic characteristics, while part 2 discloses the physicochemical properties of 25 selected protein soft drinks.

2.3.1. Part 1: Retail Market analysis

2.3.1.1. Beverage descriptions

From the evaluated protein enriched soft drinks, majority of beverages were found on sport, fitness nutrition type websites (92%). The product descriptions were provided from the product website. Retailers' names are given in **Supplementary Table 2.1**. According to the product description, these beverages are targeted towards consumers with an active lifestyle such as athletes and sport enthusiasts. The products are marketed as a refreshing or hydrating pre- and/or post-workout sport drinks with the offered added benefit of a high dose of protein. While some products contain additives for additional enhancement such as caffeine, others also advertise to support the production of hair, skin, and nail growth advertised as a nutricosmetic or beauty drink (8%).

2.3.1.2. List of Ingredients

An overview of the ingredients of the 138 different protein soft drinks are illustrated in **Table 2.1.** The base of all drinks was water enriched with, an artificial or natural colouring and a flavouring. The most frequently used non-calorific sweetener was sucralose (in 57% of protein soft drinks), followed by steviol glycosides (in 38% of protein soft drinks) and acesulfame potassium (in 14% of protein soft drinks). In some protein soft drinks sugar alcohols, such as erythritol or inositol, were used as sweeteners. These sweeteners were commonly used in combination. Calorific sweeteners, such as sucrose and fructose, were found in minority of drinks (7%). Acidulants are added in soft drinks to enhance the flavour as well as act as a preservative. The acidulants predominantly used are citric acid, phosphoric acid and malic acid, often added in combination. Potassium sorbate, potassium benzoate and sodium benzoate are preservatives present in the protein soft drinks to prevent microbial spoilage and, thus, to prolong shelf life. However, 57% of protein soft drinks did not contain any preservatives (12% from PBP drinks and 44% from animal-based drinks). Protein ingredients were commonly added as the second ingredient on the list. Both animal and plant-based sources were found amongst the 138 protein soft drinks. The protein ingredients included the branch chain amino acids (BCAA), beef protein isolate (BPI), collagen protein/hydrolysate (CP/H), milk protein concentrate/isolate (MPC/I), pea protein/isolate/hydrolysate (PP/I/H) and whey protein isolate/hydrolysate (WPI/H). The carbonated beverages only differed by the addition of CO_2. Other food additives, including stabilizers, emulsifiers and antifoaming agents were found in both carbonated and

uncarbonated beverages, displayed in **Table 2.1**. Silicone based antifoaming agents were more prevalent amongst the carbonated beverages, used to prevent foaming caused by protein ingredients. Hydrocolloids were added which act as stabilizers to enhance viscosity and mouthfeel of a beverage and also stabilize incorporated CO_2 gas cells in the carbonated beverages. Other ingredients including vitamins, minerals, botanicals and added caffeine were included in the products to enhance the nutritional benefits as well for a physiological purpose that can be more appealing to the consumer. Additionally, caffeine is added as a flavouring when stated immediately after the term, flavouring(s) in the ingredients list (European Parliament & Council, 2011).

Table 2.1: Summarized ingredient overview of carbonated and uncarbonated protein enriched soft drink formulations and the number of beverages containing each ingredient indicated in brackets (n = 138).

Protein		**Sweeteners**		**Acidulants**		**Preservatives**		**Stabilisers**	
Carbonated	*Uncarbonated*	*Carbonated*	*Uncarbonated*	*Carbonated*	*Uncarbonated*	*Carbonated*	*Uncarbonated*	*Carbonated*	*Uncarbonated*
WPI (10)	BPI (5)	Sucralose (23)	Sucralose (55)	Citric acid (27)	Citric acid (54)	Potassium	Potassium	Gum Arabic (1)	Maltodextrin (7)
WPH (3)	WPI (67)	Acesulfame-K	Steviol glycosides	Malic acid (8)	Phosphoric acid (53)	sorbate (7)	sorbate (20)	Glycerol ester	Glycerol ester of
CH (Bovine) (2)	CH (Bovine) (13)	(5)	(45)	Phosphoric acid (4)	Malic acid (26)	Sodium	Sodium	of wood rosin	wood rosin (4)
CH (Marine) (5)	CH (Marine) (1)	Steviol	Acesulfame-K (15)	Potassium citrate (4)	Sodium citrate (18)	benzoate	benzoate (16)	(1)	Modified food
CH (unknown	CP/H (unknown source) (4)	glycosides (7)	Erythritol (13)	Sodium citrate (2)	Sodium phosphate	(6)	Potassium	Pectin (1)	starch (4)
source) (3)	MPI (1)	Fructose (2)	Cane sugar (7)	Magnesium citrate	(9)		phosphate (6)	Cellulose gum	Inulin (3)
PPH (4)	MPC (1)	Inositol (1)	Saccharin (1)	(1)	Lactic acid (1)		Potassium	(1)	Pectin (1)
BCAA (8)	PP (4)		Fructose (1)				benzoate (3)		Acacia gum (1)
	PPH (2)						Calcium citrate		
	PPI (3)						(2)		
	BCAA (2)								
	Glutamine (5)								

Emulsifiers		**Antioxidants**		**Anti-foaming agents**	
Carbonated	*Uncarbonated*	*Carbonated*	*Uncarbonated*	*Carbonated*	*Uncarbonated*
Glycerol (5)	Polysorbate 80 (4)	Ascorbic acid (2)	Ascorbic acid (8)	Silicone (5)	Dimethylpolysiloxane (1)
	Glycerine (3)				
	Lecithin (2)				

WPI/H; Whey protein isolate/hydrolysate, BPI; Beef protein isolate, CP/H; Collagen protein/hydrolysate, MPC/I; Milk protein concentrate/isolate, PPI/H; Pea protein/isolate/hydrolysate, BCAA; Branch chain amnio acids

2.3.1.3. Nutritional dimension

The nutritional properties of the selected protein enriched soft drinks were analysed. The protein source, the country of origin, the price per 100 ml, and claims on the label are demonstrated in **Figure 2.1**. Whey protein isolate (WPI) was mostly used as a protein ingredient amongst the assessed beverages for both carbonated (29% of the beverages) and non-carbonated (62% of the beverages). The second most used protein ingredient was collagen hydrolysate (CH) originated from marine and bovine sources. Beef protein isolate (BPI), Pea protein isolate (PPI), Pea protein hydrolysate (PPH), the branched chain amino acids (BCAAs), such as leucine, isoleucine, and valine, and glutamine were also used. The nutritional tables on the products were assessed and summarised in ranges per 100 ml. The calorie content for the majority of the beverages (77%) was low according to EU regulation of less than 20 kcal per 100ml, ranging from 0 to 41 kcal per 100 ml (European Parliament & Council, 2006). The protein content ranged from 0.9 to 6.8%, where the average protein content used for a carbonated soft drink was 3% and for an uncarbonated it was found at 3.6%. The most frequently used protein content for both carbonated and uncarbonated was 4%. The highest protein content was found in an uncarbonated beverage using WPI, followed by an uncarbonated soft drink with BPI containing 6% protein. Carbonated protein soft drinks included lower amounts of proteins compared to uncarbonated drinks. The highest protein content found amongst the carbonated beverages was 5.8%, followed by 4.2%, both using WPI. The lowest protein content found for uncarbonated was at 1% using glutamine and for carbonated 0.9% using BCAAs.

Only 18% of the 138 selected protein soft drinks contained plant-based sources of protein. The protein sources used were PPI, PPH, BCAAs and glutamine (sourced from the by-processing of corn). The highest protein content amongst the PBP soft drinks was 4.6% in an uncarbonated beverage which used PPI and the lowest content was found in a carbonated beverage at 0.9% using BCAAs.

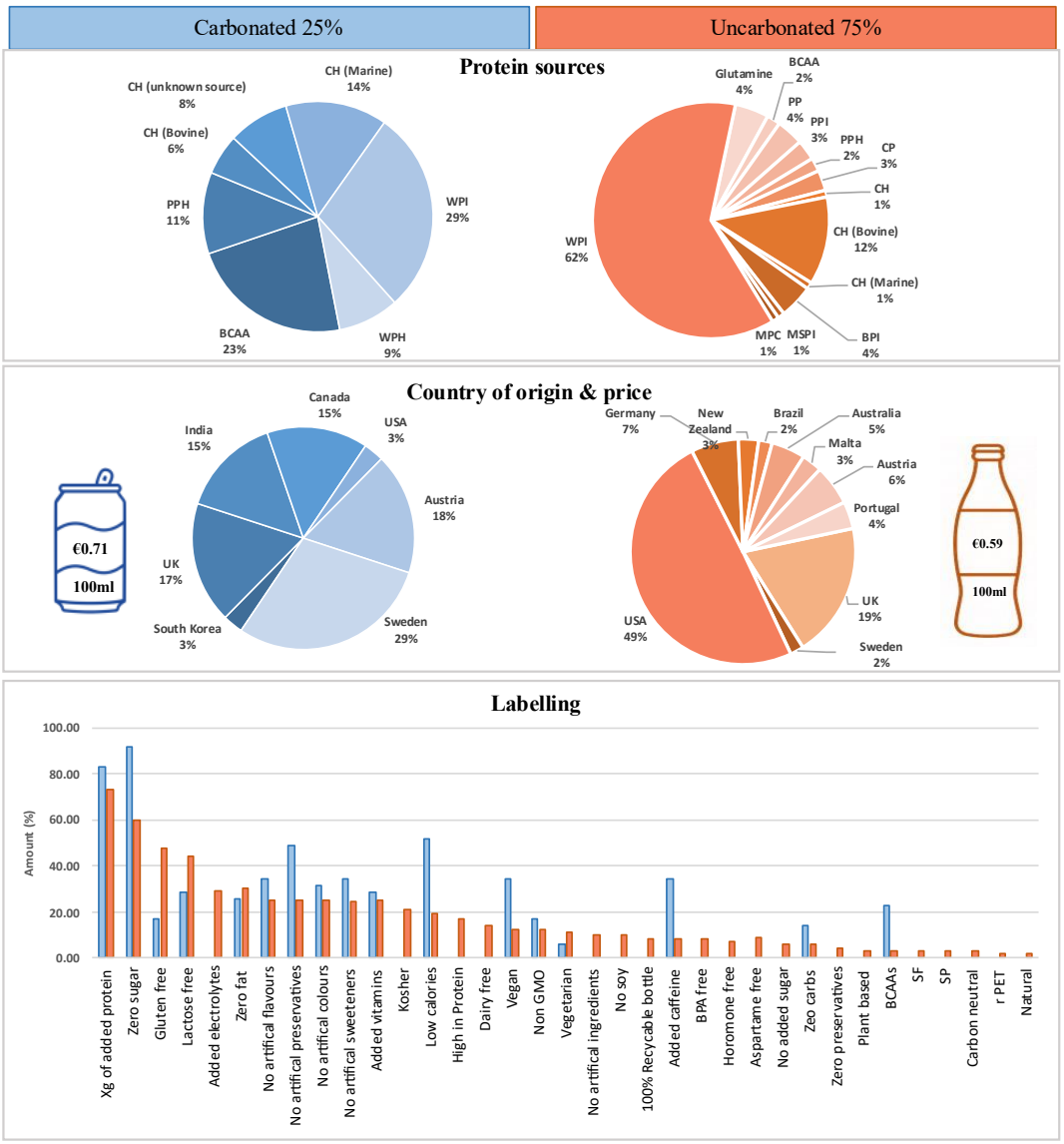

Figure 2.1: Overview of market analysis of commercially available high protein soft drinks.

2.3.1.4. Economic dimension

The country of origin, price and labelling/marketing was assessed and are displayed in **Figure 2.1**. The analysed beverages were sourced over 13 different countries, (Austria, Australia, Brazil, Canada, Germany, India, Portugal, Malta, New Zealand, South Korea, Sweden, UK and the USA). The US dominated the market for uncarbonated soft drinks making 50% of the 138 selected beverages, followed by the UK with 19%. Sweden dominated the market for carbonated soft drinks (29% of 138 selected beverages), followed by Austria and the US. The average cost of a carbonated drink was €0.71 per 100 ml and for uncarbonated €0.59 per 100 ml, where the product was typically more expensive when the product contained a higher protein content. The protein beverages were marketed with specific claims and labels. Most protein soft drinks contained a label stating the amount of protein in grams and ‘zero added

sugar'. Specifically for the carbonated drinks, labels/claims such as 'low calories', 'no artificial preservatives', 'vegan' and 'added caffeine' were most frequently used. On the uncarbonated beverages, the labels/claims 'gluten free' and 'lactose free' were amongst the most popular.

2.3.2. Part 2: Protein content and physicochemical properties of selected protein soft drinks

The protein content and the physicochemical properties of 25 selected protein beverages were determined. This included appearance and colour, protein content, pH and TTA, apparent viscosity, density, particle size of the dispersion and dispersion stability.

2.3.2.1. Pictures and colour

The colour CIE L* a* b* values of each beverage are shown in **Table 2.2**. Pictures of each soft drinks are displayed in **Figure 2.2** with corresponding RGB colour results. Measuring the product colour is important for product consistency and any deviations from the expected colour could pose quality issues. All beverages obtained high L* values and very white to clear colours, where the darkest soft drink obtained an L* value of 61 ± 0.42 showing a brown shaded colour.

Table 2.2: Nitrogen content and physicochemical properties of protein enriched soft drinks. Soft drink (SD), numbers 1-11 indicate product brand and the corresponding letter (a – d) shows different flavours of the same brand. An *—carbonated. Values with the same superscript letters are not significantly different ($p < 0.05$).

Soft drink	Nitrogen (%)	Particle size (nm)	Polydispersity index	Separation rate (%/min)	L*	a*	b*
SD 1.a	0.94 ± 0.01 ^{a}	3359 ± 498 ^{a}	0.26 ± 0.1 ^{ac}	0.198 ± 0.0 ^{a}	69.49 ± 0.11 ^{a}	12.14 ± 0.02 ^{a}	29.67 ± 0.03 ^{a}
SD 2.a	0.53 ± 0.0 ^{b}	50 ± 1.37 ^{b}	0.41 ± 0.02 ^{bdef}	0.07 ± 0.0 ^{bcef}	64.88 ± 0.18 ^{b}	40.69 ± 0.11 ^{b}	3.02 ± 0.09 ^{b}
SD 3.a*	0.64 ± 0.0 ^{e}	104 ± 2.18 ^{bcd}	0.4 ± 0.04 ^{bdef}	0.064 ± 0.01 ^{cefg}	86.4 ± 0.09 ^{c}	1.26 ± 0.04 ^{c}	17.39 ± 0.0 ^{c}
SD 3.b*	0.64 ± 0.0 ^{e}	117 ± 2.44 ^{bcd}	0.36 ± 0.04 ^{bdef}	0.08 ± 0.01 ^{bc}	88.55 ± 0.13 ^{p}	0.85 ± 0.03 ^{d}	15.53 ± 0.03 ^{d}
SD 3.c*	0.65 ± 0.01 ^{efg}	104 ± 2.18 ^{bcd}	0.4 ± 0.04 ^{bcd}	0.036 ± 0.01 ^{fgh}	90.11 ± 0.03 ^{r}	0.53 ± 0.01 ^{s}	14.8 ± 0.03 ^{e}
SD 4.a	0.6 ± 0.0 ^{c}	54 ± 2.09 ^{b}	0.35 ± 0.05 ^{bcd}	0.102 ± 0.01 ^{bd}	97.12 ± 0.01 ^{d}	-0.96 ± 0.03 ^{e}	8.33 ± 0.0 ^{f}
SD 4.b	0.59 ± 0.01 ^{c}	65 ± 1.81 ^{bc}	0.42 ± 0.04 ^{bdef}	0.066 ± 0.01 ^{bcef}	89.11 ± 0.02 ^{q}	10.44 ± 0.01 ^{u}	4.24 ± 0.02 ^{v}
SD 5.a	0.37 ± 0.0 ^{d}	59 ± 1.08 ^{b}	0.46 ± 0.02 ^{defg}	0.038 ± 0.01 ^{efgh}	88.58 ± 0.02 ^{p}	11.42 ± 0.02 ^{f}	5.96 ± 0.02 ^{w}
SD 5.b	0.36 ± 0.01 ^{d}	160 ± 6.07 ^{bcde}	0.63 ± 0.04 ^{h}	0.036 ± 0.02 ^{fgh}	89.79 ± 0.06 ^{r}	2.43 ± 0.02 ^{g}	11.3 ± 0.02 ^{g}
SD 5.c	0.36 ± 0.01 ^{d}	68 ± 4.04 ^{bc}	0.56 ± 0.01 ^{gh}	0.026 ± 0.01 ^{h}	98.13 ± 0.02 ^{e}	-0.26 ± 0.04 ^{h}	3.27 ± 0.03 ^{h}
SD 6.a	0.59 ± 0.01 ^{c}	6 ± 1.14 ^{b}	0.35 ± 0.06 ^{bcd}	0.064 ± 0.0 ^{cefg}	96.69 ± 0.01 ^{s}	-2.35 ± 0.01 ^{i}	17.08 ± 0.03 ^{i}
SD 6.b	0.64 ± 0.0 ^{ef}	6 ± 0.41 ^{b}	0.35 ± 0.02 ^{bcd}	0.078 ± 0.01 ^{bc}	96.58 ± 0.04 ^{s}	-5.88 ± 0.03 ^{j}	24.87 ± 0.05 ^{j}
SD 6.c	0.65 ± 0.0 ^{efg}	13 ± 5.74 ^{b}	0.51 ± 0.1 ^{fg}	0.064 ± 0.0 ^{cefg}	89.35 ± 0.01 ^{q}	10.47 ± 0.02 ^{u}	7.79 ± 0.03 ^{k}
SD 6.d	0.66 ± 0.0 ^{fg}	11 ± 1.67 ^{b}	0.66 ± 0.09 ^{h}	0.056 ± 0.0 ^{cefgh}	97.59 ± 0.0 ^{f}	0.34 ± 0.0 ^{r}	6.12 ± 0.01 ^{w}
SD 7.a	0.66 ± 0.0 ^{g}	791 ± 63.89 ^{h}	0.49 ± 0.16 ^{bde}	0.068 ± 0.01 ^{cefgh}	66.27 ± 0.04 ^{g}	15.88 ± 0.05 ^{k}	31.02 ± 0.02 ^{l}
SD 7.b	0.65 ± 0.0 ^{efg}	713 ± 42.5 ^{hi}	0.39 ± 0.06 ^{efg}	0.118 ± 0.01 ^{d}	76.14 ± 0.25 ^{h}	17.4 ± 0.04 ^{l}	10.75 ± 0.04 ^{m}
SD 8.a	0.51 ± 0.02 ^{b}	133 ± 2.6 ^{bcd}	0.4 ± 0.05 ^{bcd}	0.028 ± 0.0 ^{gh}	88.27 ± 0.02 ^{p}	0.57 ± 0.04 ^{s}	12.77 ± 0.05 ^{n}
SD 8.b	0.52 ± 0.0 ^{b}	185 ± 2.73 ^{bcde}	0.32 ± 0.01 ^{bc}	0.028 ± 0.01 ^{gh}	89.15 ± 0.01 ^{q}	9.12 ± 0.01 ^{t}	15.71 ± 0.01 ^{o}
SD 9.a*	0.29 ± 0.0 ^{h}	492 ± 11.2 ^{fg}	0.18 ± 0.03 ^{a}	0.264 ± 0.01 ^{i}	61.08 ± 0.05 ^{i}	3.68 ± 0.02 ^{m}	21.34 ± 0.05 ^{p}
SD 9.b*	0.3 ± 0.0 ^{h}	271 ± 4.7 ^{de}	0.2 ± 0.02 ^{a}	0.086 ± 0.01 ^{bcd}	87.1 ± 0.01 ^{j}	9.38 ± 0.02 ^{n}	8.52 ± 0.07 ^{q}
SD 10.a*	0.3 ± 0.0 ^{h}	735 ± 74.57 ^{h}	0.47 ± 0.09 ^{efg}	0.156 ± 0.01 ^{j}	85.27 ± 0.08 ^{k}	-1.32 ± 0.02 ^{o}	20.29 ± 0.02 ^{r}
SD 10.b*	0.3 ± 0.0 ^{h}	950 ± 34.14 ^{i}	0.32 ± 0.07 ^{bc}	0.202 ± 0.0 ^{a}	60.97 ± 0.42 ^{l}	9.23 ± 0.1 ^{t}	26.15 ± 0.2 ^{s}
SD 11.a*	0.11 ± 0.0 ^{i}	325 ± 12.6 ^{efg}	0.34 ± 0.05 ^{bc}	0.17 ± 0.01 ^{aj}	85.85 ± 0.08 ^{m}	0.32 ± 0.01 ^{r}	2.54 ± 0.02 ^{t}
SD 11.b*	0.1 ± 0.0 ^{i}	400 ± 8.9 ^{fg}	0.2 ± 0.01 ^{a}	0.074 ± 0.01 ^{bce}	78.02 ± 0.03 ^{n}	20.8 ± 0.03 ^{p}	14.09 ± 0.04 ^{u}
SD 11.c*	0.11 ± 0.0 ^{i}	245 ± 55.39 ^{cdef}	0.42 ± 0.08 ^{bdef}	0.064 ± 0.01 ^{cefg}	95.62 ± 0.01 o	4.48 ± 0.04 ^{q}	4.37 ± 0.03 ^{v}

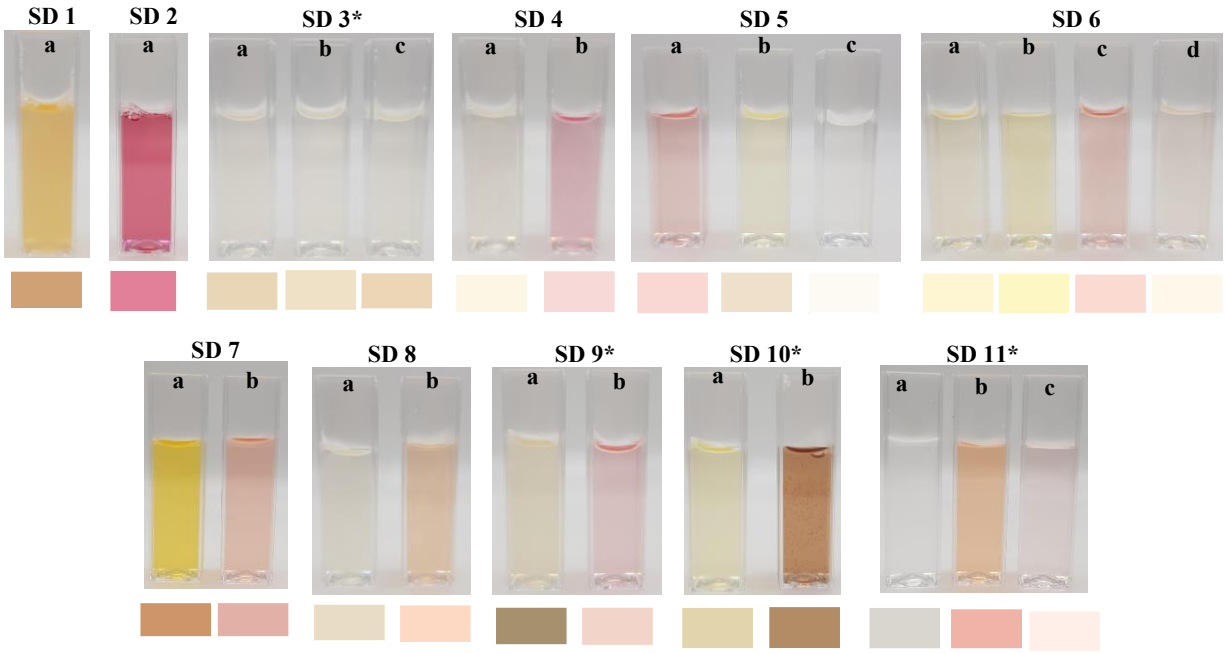

Figure 2.2: Images of all soft drink samples with CIE RGB generated values. Soft drinks with * carbonated.

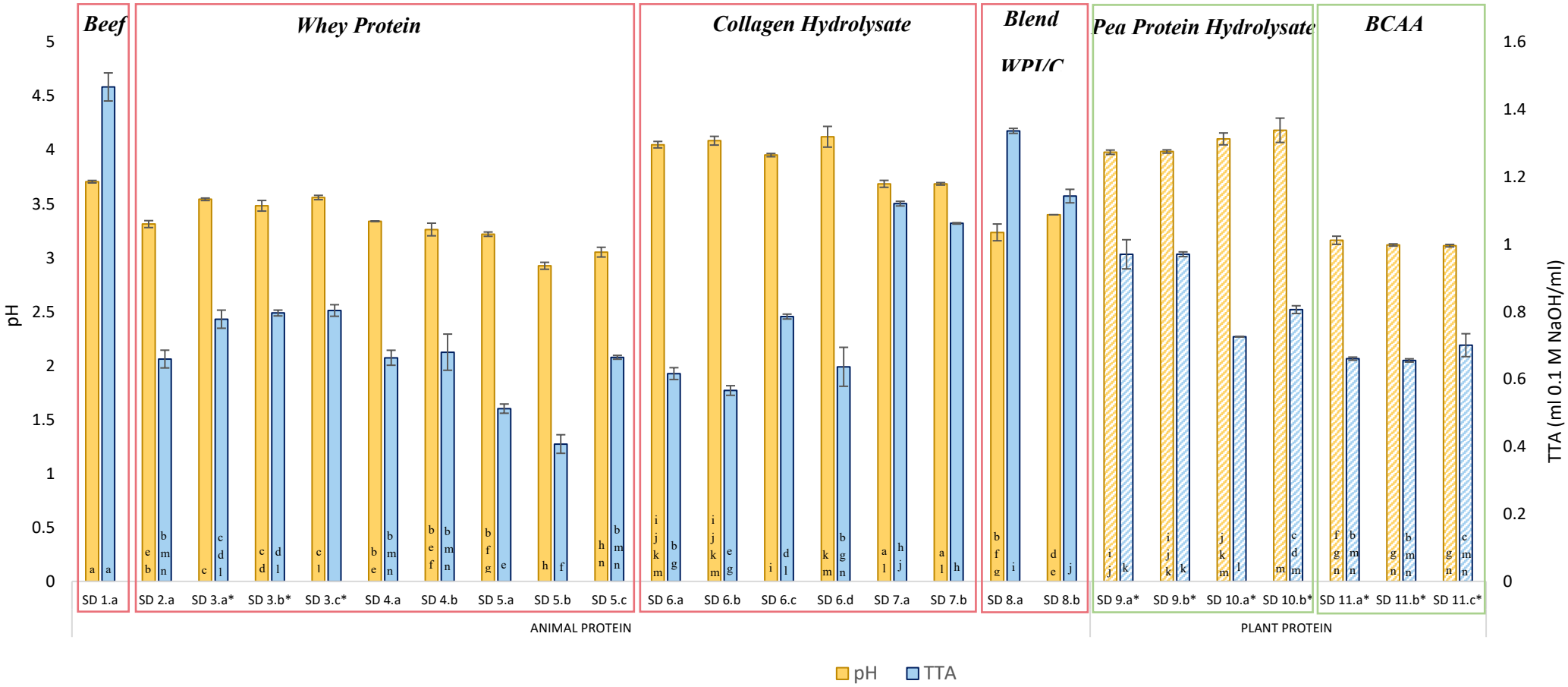

Figure 2.3: pH and TTA , distinguished into animal and plant-based sources of protein, no pattern bars show animal protein, and bars with pattern show plant-based protein. Soft drink (SD), numbers 1–11 indicate product brand and the corresponding letter (a–d) shows different flavours of the same brand. An *—carbonated. Values with the same superscript letters are not significantly different ($p < 0.05$).

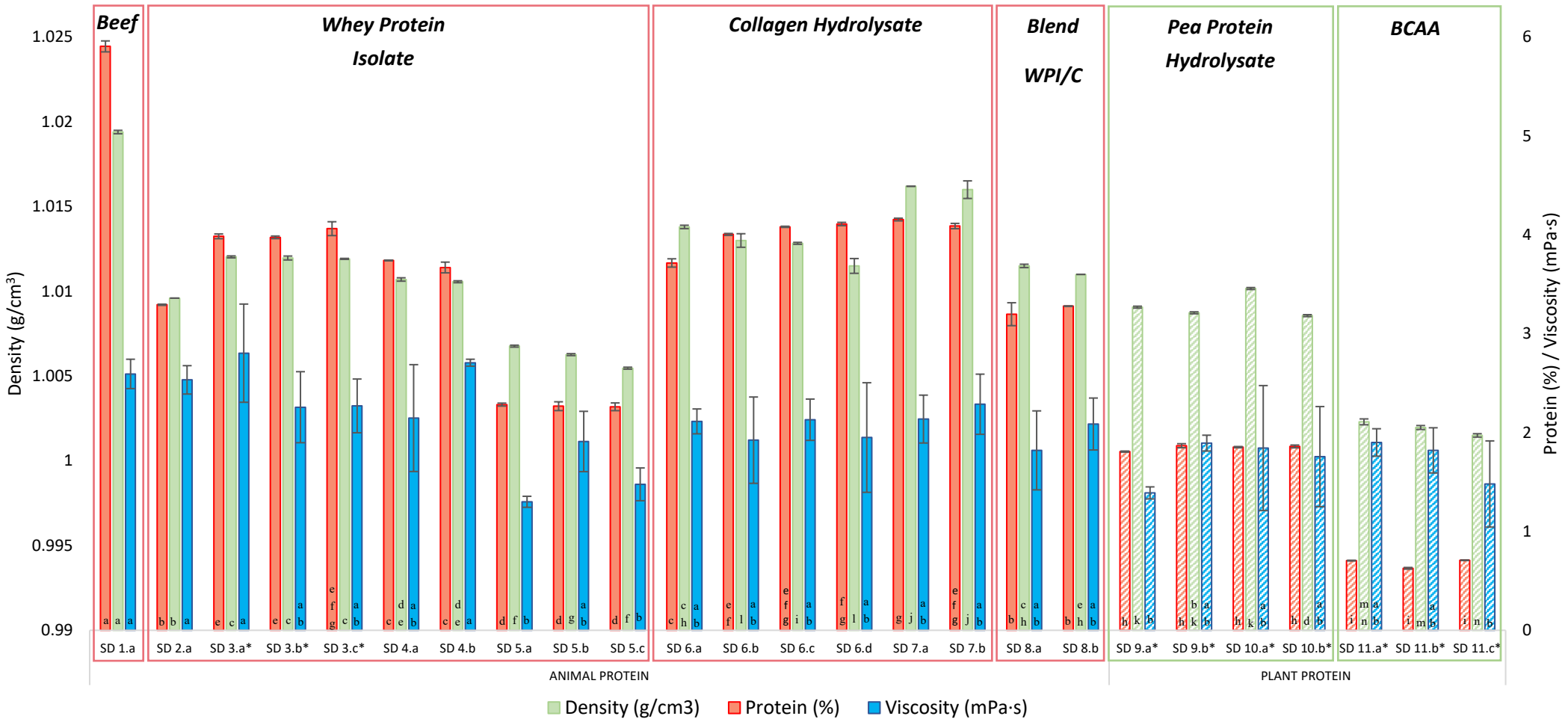

Figure 2.4: Protein content (determined using the Kjeldahl method and using a nitrogen conversion factor of 6.25), apparent viscosity and density of commercial high protein soft drinks, distinguished into animal and plant-based sources of protein, no pattern bars show animal protein, and bars with pattern show plant-based protein. Soft drink (SD), numbers 1–11 indicate product brand and the corresponding letter (a–d) shows different flavours of the same brand. An *—carbonated. Values with the same superscript letters are not significantly different ($p < 0.05$).

2.3.2.2. pH and total titratable acidity (TTA)

The pH and TTA of the beverages are displayed in **Figure 2.3**. The pH of all beverages showed acidic values which ranged from 2.9 to 4.2. The highest pH value was found in SD 10.b* followed by SD 6.d, SD 10.a* and SD 6.b and SD 6.a. The lowest pH value was found in SD 5.b followed by SD 5.c. SD 1.a displayed the highest TTA value (1.47 ± 0.04 ml of 0.1 M NaOH/ml) followed by SD 8, SD 7 and SD 9* ranging from 1 ml to 1.3 ml 0.1 M NaOH/ml. SD 5.b showed the lowest TTA value (0.4 ± 0.03 ml) of 0.1M NaOH/ml).

2.3.2.3. Protein content

The protein content of the beverages is shown in **Figure 2.4**. All beverages had protein contents similar to the product label, except for SD 2. This particular beverage was labelled at 4 g/100 ml derived from WPI and was found to have a protein content of 3.3 % ± 0.01. SD 1.a showed the highest protein content originated from BPI at 5.91 % ± 0.06, labelled as 6%.

2.3.2.4. Apparent viscosity

The formulation of a product can potentially impact viscosity which is an important parameter capable of influencing the mouthfeel of beverages. A suitable viscosity provides desirable and consistent mouthfeel that can enhance the sensory experience for the consumer. Shown in **Figure 2.4**, SD 3.a* exhibited the highest viscosity (2.8 ± 0.5 mPa·s) similar to SD 4.b and SD 1.a at 2.71 ± 0.04 mPa·s and 2.59 ± 0.15 mPa·s respectively. SD 5.a showed the lowest viscosity at 1.3 ± 0.06 mPa·s followed by SD 9.a* and SD 5.c at 1.39 ± 0.06 mPa·s and 1.48 ± 0.17 mPa·s respectively. The remaining beverages showed a viscosity range between 1.48 and 2.53 mPa·s.

2.3.2.5. Density

Density is an important physical parameter that indicates the soluble solids of a soft drink. The density of a solution is dependent on the concentration of dissolved substances within the product where water contains an average density value of 1 g/cm^3. SD 1.a showed a significantly higher density value displayed in **Figure 2.4** compared to all other brands at 1.0194 ± 0.0 (g/cm^3). This was followed by the brand SD 7, both flavours at 1.016 ± 0.0 g/cm^3. SD brand 11* showed significantly lower density values at 1.0015 ± 0.0 g/cm^3, 1.0019 ± 0.0 g/cm^3 and 1.0054 ± 0.0 g/cm^3 from flavour b, a and c, respectively.

2.3.2.6. Particle size and polydispersity index (PDI)

The particle size of the dispersion within a beverage system can affect certain attributes of the product such as mouthfeel, taste and dispersion stability. The particle size in nm and polydispersity index are shown in **Table 2.2**. SD 1.a obtained a significantly larger particle size at 3359 ± 498 nm compared to all other products. SD 10* and SD 7 brands obtained similar particle sizes at 950 ± 34.14 nm and 736 ± 74.57 nm for SD 10* b and a and 791 ± 63.89 nm and 713 ± 42.5 nm for SD 7 b and a, respectively. The smallest particle size was found in the brand SD 6 at 6 nm for both a and b flavour followed by 11 ± 1.67 nm and 13 ± 5.74 nm for d and c flavour, respectively. The remaining beverages obtained a particle size ranging from 51 to 492 nm. Regarding PDI, SD 6.d obtained the highest value at 0.66 ± 0.09. while SD 9.a* showed the lowest PDI (0.18 ± 0.03). Majority of the samples obtained a PDI < 0.5 (18 samples).

2.3.2.7. Stability

The separation rate of each sample is presented in **Table 2.2**. Overall results indicate that all beverages are shown to be stable according to transmission profile Lumisizer graphs (shown in **Supplementary Figure 2.1**). SD 9.a* separated the fastest at a separation rate of 0.264 ± 0.01 %/min followed by SD 10.b* and SD 1.a at 0.202 ± 0.0 %/min and 0.198 ± 0.0 %/min, respectively. The lowest separation rate occurred for SD 5.c at 0.026 ± 0.01 %/min. SD 8 brand showed the next slowest separation rate for both flavours (0.028 %/min). Sedimentation layers were found on some products which can be seen from the displayed Lumisizer graph (i.e., the build-up of red and green lines at right hand side of graph shown in **Supplementary Figure 2.1**) due to insoluble particles. However, no sample experienced any flocculation/creaming.

2.3.2.8. Principal Component Analysis biplot

Principal component analysis (PCA) biplot was performed on the average beverage results, (**Figure 2.5**) and shows the different types of correlations at 65.44%. Some brands can be discriminated from other brands. SD 1.a is separated from all other brands, classed by its high particle size, TTA and density values. SD 9.a* is also distinguished from other brands shown by its fast separation rate and low PDI. Brands SD 5, 8, 10* and 11* are clearly grouped indicating similarities. Protein content with viscosity (*p*-value: 1.67E-04, *r*-value: 0.69), protein content with density (*p*- value: 9.6E-11, *r*-value: 0.93), and density with viscosity (*p*-value: 2.03E-03, *r*-value: 0.60) all showed a significantly strong positive correlation with one

another. PDI and separation rate are shown to be independent with a negative correlation, but this was not significant (*p*- value: 0.02, *r*-value: - 0.48). The remaining groups show no significant or correlation between them.

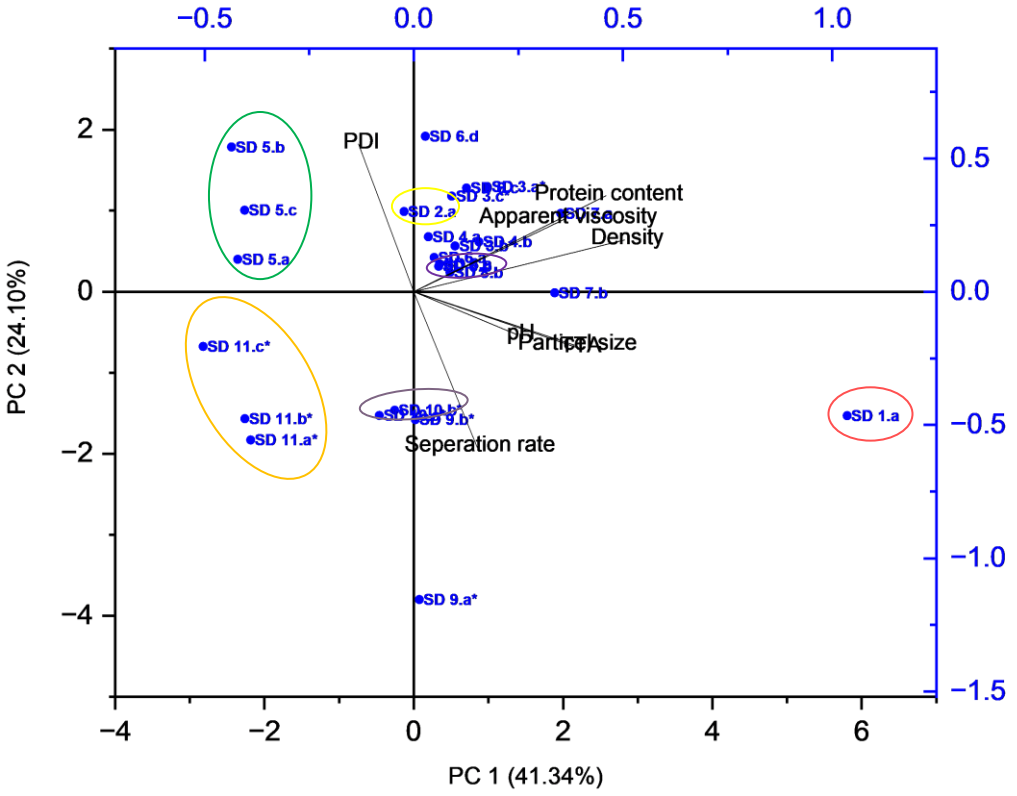

Figure 2.5: Principal Component Analysis biplot of commercial high protein soft drinks (SD) with * carbonated, numbers 1-11 indicate product brand and the corresponding letter (a-d) shows different flavours of the same brand.

2.4. Discussion

This study focuses on the comprehensive retail market analysis of commercial high protein soft drinks including the assessment of the nutritional and compositional characteristics as well as the physicochemical and functional properties of selected beverages. Based on the market analysis, WPI was the most popular added protein ingredient. This can be attributed to its excellent protein quality, as WPI has a digestible indispensable amino acid score (DIAAS) of > 100% (Herreman et al., 2020). According to the FAO a DIAAS of > 100%, > 75% and < 75% indicates an excellent, good, and poor-quality protein, respectively (FAO, 2013). Moreover, WPI exhibits high solubility over a range of pH values including acidic conditions due to the electrostatic repulsion of positively charged ions below its isoelectric point which is ~ pH 5 (Goulding et al., 2019; Tang et al., 2023). Additionally, this ingredient has been shown to provide low turbidity and absence of colour which delivers a favourable aspect to a soft drink (Prado et al., 2015). Similar to WPI, beef protein is also regarded as a nutritionally excellent quality protein and was used amongst certain products (FAO, 2013). CH was identified as the second most utilised protein ingredient. There are many different types of CH's including marine and bovine sources, as discovered in the market analysis. Unidentified sources of this ingredient can include alternatives such as porcine or poultry CH (León-López et al., 2019). CH might appeal to consumers due to its associated health and cosmetic benefits as well as for industries advantage, as it's also very soluble in acidic media (León-López et al., 2019). A downfall for CH is that it has a DIAAS score of 0 due to the lack of the essential amino acid (EAA) tryptophan and contains mostly non-EAA with glycine being the most abundant followed by proline (Gauza-Włodarczyk et al., 2017; Phillips, 2017). The market analysis revealed that PBP soft drinks only accounted for 18% of the total market and offered limited sources of plant proteins. Soft drinks with animal-based protein showed the highest protein contents. The lowest protein contents were found with PBPs, where the inclusion of these proteins to this type of beverage can pose some challenges such as poor solubility, off flavours, limiting amino acids and anti-nutrients, and the consideration of digestibility or bioavailability (Bessada et al., 2019; Gorissen et al., 2018; Ma et al., 2022). Amongst the remaining products, lack of complete EAA profiles were found due to products only including BCAA's and the single amino acid glutamine. PPI/H obtains a DIAAS score > 75% due to its limiting amino acids methionine and cysteine.

To achieve a DIAAS > 100%, blending two or more plant-based proteins such as a legume and cereal protein can provide a complete EAA profile (Herreman et al., 2020). Enriching beverages with PBP is expanding which is especially being seen within carbonated beverages to produce protein rich drinks (Arbach et al., 2021). With increasing growth in global population and increasing protein demand on the rise, PBP's are preferred to animal-based protein from an environmental perspective (Henchion et al., 2017). PBP's are associated with lower land use and lower production of greenhouse gases, having less of an impact on climate change (Henchion et al., 2017). Individuals who substitute animal-based proteins with PBP ought to consider the potential deficiency in complete amino acid profiles from a health standpoint. These beverages are targeted towards consumers looking to increase their protein intake in a convenient way mainly as a pre- or post-workout drink that is known to improve the recovery and growth of muscle (Schoenfeld et al., 2017). The display of grams of protein per serving on the front of the product is important for the consumer to emphasis its significance, as it was the most popular label used.

The ingredient list of the beverages showed little variations. Non calorific or artificial sweeteners were predominantly used. Health conscious and sport driven consumers aim to reduce their sugar consumption and expect low or calorie free products (Julian Mellentin, 2022). According to one of the 10 key trends in the New Nutrition Business report by Julian Mellentin, (2022) sweetness reinvented, forecasted for 2023, industry is reformulating products with non-calorific sweeteners to have the ability to add the "zero added sugar" label (Julian Mellentin, 2022). Additionally, the introduction of the sugar taxes further increased this trend, where beverage companies reduce added sugar to keep their products at reasonable prices for the consumer (Popkin & Hawkes, 2016; Thow et al., 2022). Furthermore, to make a high protein claim on a food label, 20% of the calories must be provided by protein (European Parliament & Council, 2006). Therefore, by lowering the calorie content, it becomes feasible to make this claim more attainable. Sucralose was the most abundant sweetener, and it is commonly used amongst the beverage industry due to its high stability at a low pH and high temperature and it obtains a sweetness profile similar to sucrose (Abu-Reidah, 2019; Edwards et al., 2016). Citric and phosphoric acid were the most abundant acidulants added. Citric acid is associated with citrus flavour and is commonly used in lemon, lime and orange flavoured beverages (Abu-Reidah, 2019). Antifoaming agents were used mainly amongst the carbonated beverages. It is permitted to use 10 mg/L of dimethyl polysiloxane (PDMS) to reduce foam in processing due to gushing which is the rapid release of CO_2. The presence of an antifoam in

carbonated products can exacerbate gushing by reducing surface tension of the product and accelerating the release of dissolved CO_2 (Ashurst et al., 2017). This was seen in a study by Prado et al. (2015), where 0.005% of PDMS was added to a carbonated orange soft drink fortified with WPI. WPI has high foaming capacity that can be induced by the introduction of carbonation (Tang et al., 2023). It can be noted that even though some beverages stated on the label the addition of anti-foaming agents, according to EU regulation, antifoamers can be considered processing aids, which are not required to be listed on the list of ingredients (European Parliament & Council, 2011). In 2014, the United States was the highest leading consumer of sugar-sweetened soft drinks, establishing its dominance in the market based on the country of origin of assessed protein beverages (Popkin & Hawkes, 2016).

The market analysis provided a basic understanding about protein soft drinks. Following, 25 protein rich soft drinks were selected and analysed regarding their protein content and physicochemical properties. Protein content of the beverages was determined using the Kjeldahl method with a conversion factor of 6.25. Other protein determination methods such as the Dumas also use conversion factors to calculate nitrogen to protein values. All beverages showed similar values when compared to their product labels with the exception of SD 2. The conversion factor 6.25 is regularly applied in industry to all proteins (animal and plant-based sources) based on the assumptions that all proteins have a nitrogen content of 16% as well as assuming all nitrogen is derived from protein. But a study by Krul (2019) reveals that this conversion factor overestimates the protein content of most foods due to variations in amino acid profiles and non-protein nitrogen where the use of conversion factors is not standardised. Therefore, nitrogen values were also reported shown in **Table 2.2**. Furthermore, some beverages use individual amino acids and incomplete proteins where proteins are defined as polymers of amino acids linked via α-peptide bonds (Watford & Wu, 2018). The use of the BCAA's in these beverages are of specific importance to athletes because these are the amino acids which extensively contribute to muscle growth and recovery (Shimomura et al., 2004).

The pH values ranged from 2.9 – 4.2 which is similar to that in literature for protein enriched soft drinks and juices (Goudarzi et al., 2015; Prado et al., 2015; Yadav et al., 2016). This can be explained by the ingredients used in the soft drinks such as the concentration of acidulants as well as dissolved carbonic acid from carbonated drinks, released during decarbonation. The addition of these acidulants is essential to enhance flavour and prevent microbial spoilage (Steen & Ashurst, 2006). Protein ingredients can function as a buffer. Increased protein content has shown to increase buffering capacity, as SD 1 obtained the highest protein and TTA values,

where SD brand 11* had the lowest protein content and the lowest TTA values (Al-Dabbas et al., 2010). Moreover, the addition of long chain peptides compared to single amino acid use, could additionally be a cause for increased buffering capacity. The use of acidulants can also contribute to the high TTA (Jindal & Singh, 2010) as well as some of the acidic preservatives used such as potassium sorbate and sodium benzoate (Ashurst, 2016), which were included in SD 1, SD 7 and SD 8 obtaining higher TTA values compared to drinks with no preservatives such as SD 5 and SD 6.

Density can be affected by the amount of soluble substances within a beverage (Jabeen et al., 2022). The analysed beverages contained mainly water. The density of water is 1 g/cm^3, and therefore, any value above this can indicate dissolved substances within the product. The analysed beverages contained non-calorific sweeteners and therefore sugar does not contribute to the increase in density. Addition or replacement of sucrose with non-calorific sweeteners within a beverage system have been shown to decrease the total soluble solids or brix value. Replacing sucrose by natural (steviol glycosides) or artificial sweeteners in soft drinks reduces the total soluble solid content (brix value) as well as the viscosity (Al-Dabbas et al., 2012; Saniah et al., 2012). The increased density of the analysed SD is, presumably, due to the addition of protein. Principal component analysis (**Figure 2.5**) showed a significantly strong positive correlation between protein concentration and density values. SD 1 obtained the highest protein content and the highest density whereas the SD 11* brand obtained the lowest density and lowest protein content.

Viscosity, along with other rheological parameters, plays a crucial role as a fundamental attribute of beverages. Beverages containing PBP's showed lower apparent viscosity values, which is most likely due to a lower concentration of protein in the beverages. Protein as an ingredient contributes mainly to the solids present within these drinks. This was also seen in a study by Yadav et al. (2016) where a significant increase in apparent viscosity was shown with an increasing level of WPC to a mango based RTD protein fortified beverage. The PCA analysis strengthens the hypothesis by showing a strong positive correlation between viscosity and protein content. However, other ingredients also contributed to increased viscosity, particularly antifoaming agents. PDMS is a silicon-based compounds and is well known to increase viscosity. Therefore, different formulations contribute to the variation in viscosity results.

Different particle sizes can be a result from different processes of protein ingredients and the beverage production process including processing parameters, such as temperature, pH, and pressure. This may contribute to positive or negative effects on the mouthfeel and taste. SD 1, which includes BPI as a protein source, displayed the largest particle size compared to other soft drinks. SD 6 brand showed the smallest particle size containing CH. Smaller size proteins can be a result of increased hydrolysis that can alter the protein structure and contribute to the formation of low molecular weight peptides (Saha & Hayashi, 2001). These smaller peptides can be a characteristic of the exposure of hydrophobic amino acids, which can cause a bitter taste (Saha & Hayashi, 2001). The presence of polydispersity in colloidal dispersions can result in the destabilisation of a system (Chu et al., 1996). Specifically, it was observed that SDs with a wider range of particle sizes or high PDI exhibited higher rates of separation. Majority of the samples obtained a PDI < 0.5 (18 samples) where only one SD 6.d showed a PDI at 0.66. A PDI below 0.5 can indicate a monodisperse sample whereas above 0.7 shows samples common to a broader size distribution of particles or polydisperse (Mudalige et al., 2018).

Soft drinks are ideally requested to have a shelf life for several months or years. The presence of phase separation in beverages is undesirable indicating its instability; the faster the separation the more unstable the beverage. Highest separation rate at 0.264 ± 0.01 %/min was shown in SD 9.a*, containing the same protein type and concentration, SD 9.a* and SD 9.b* were not expected to obtain different separation rates. However, the presence of fruit particles in SD 9.a*, most likely, accelerated the samples separation. Overall, from the Lumisizer graphs, (**Supplementary Figure 2.1**) all beverages were shown to be quite stable. This can potentially be attributed to the high solubility of protein ingredients used, caused by weak electrostatic interactions and a high net charge (Cano-Sarmiento et al., 2018). For enhanced stability, a study by Zhang et al. (2023) showed the use of polysaccharide stabilizers, (beta glucan, low methyl pectin, chitosan) in a cranberry juice fortified with collagen peptides resulting in a reduction in turbidity at acidic pH values. Moreover, lower centrifugal precipitation which is an indication of stability was observed with the addition of these stabilizers at acidic pH values. SD brand 7 which uses CH was one of the analysed beverages obtaining a stabilizer (maltodextrin) in the formulation potentially to increase dispersion stability.

Beverage colour is important parameter for consumers and a quality criterion for industry. Both artificial and natural colours were added to some beverages including beta carotene, sunset yellow and carmine. Certain colours complement certain tastes such as red is linked to fruity flavours (strawberry, raspberry), orange and yellow are commonly associated with citrus

flavours and browns align with heavy flavours including colas and shandies (Ashurst, 2016). The function of adding colourants to a soft drink is for the interaction between sight and taste as the colour effects how individuals perceive the flavour (Redondo et al., 2014).

Enriching beverages with protein yields numerous advantages, including enhanced nutritional profiles of the products and targeted applications in post-exercise recovery, among other potential benefits.

2.5. Conclusions

Market research analysis of 138 protein enriched soft drinks revealed animal-based protein saturated the market where WPI and CH were the most common protein ingredients used. The market is lacking in PBP soft drinks at only 18% of surveyed beverages. These beverages offer a convenient choice for athletes and sports enthusiast who require a substantial amount of protein, crucial for the growth and repair of muscle. Lastly, complete amino acids profiles were scarce with only some drinks using single amino acids. Majority of the beverages were shown to obtain a low protein content, particularly the PBP soft drinks, where the integration of these proteins is difficult in a beverage matrix due to their physicochemical properties but also due to their unappealing associated sensory characteristics. A high protein claim addressed from EU guidelines is only possible due to the very low-calorie content of these beverages. The physiochemical analysis of the 25 selected commercial beverages showed that protein content and type had significant impact on functional properties of the beverages. This was seen where increased protein content affected density and viscosity, as well as PBP showed lower viscosity values compared to animal-based proteins with some exceptions that can also be attributed to their lower protein content. These findings provide valuable insights into the key quality characteristics of protein-enriched soft drinks and establish important connections between functional properties and formulations. This knowledge can be utilised for future product development of these soft drinks. Future research should be focused on the development of easily applicable PBP's in soft drinks with complete EAA profiles that can be incorporated by the inclusion of a blend of complementing plant proteins such as a cereal-based protein together with a legume protein. Moreover, due to climate change and the increasing environmental awareness of government and consumers, there is a high demand for plant-based product.

2.6. Acknowledgments:

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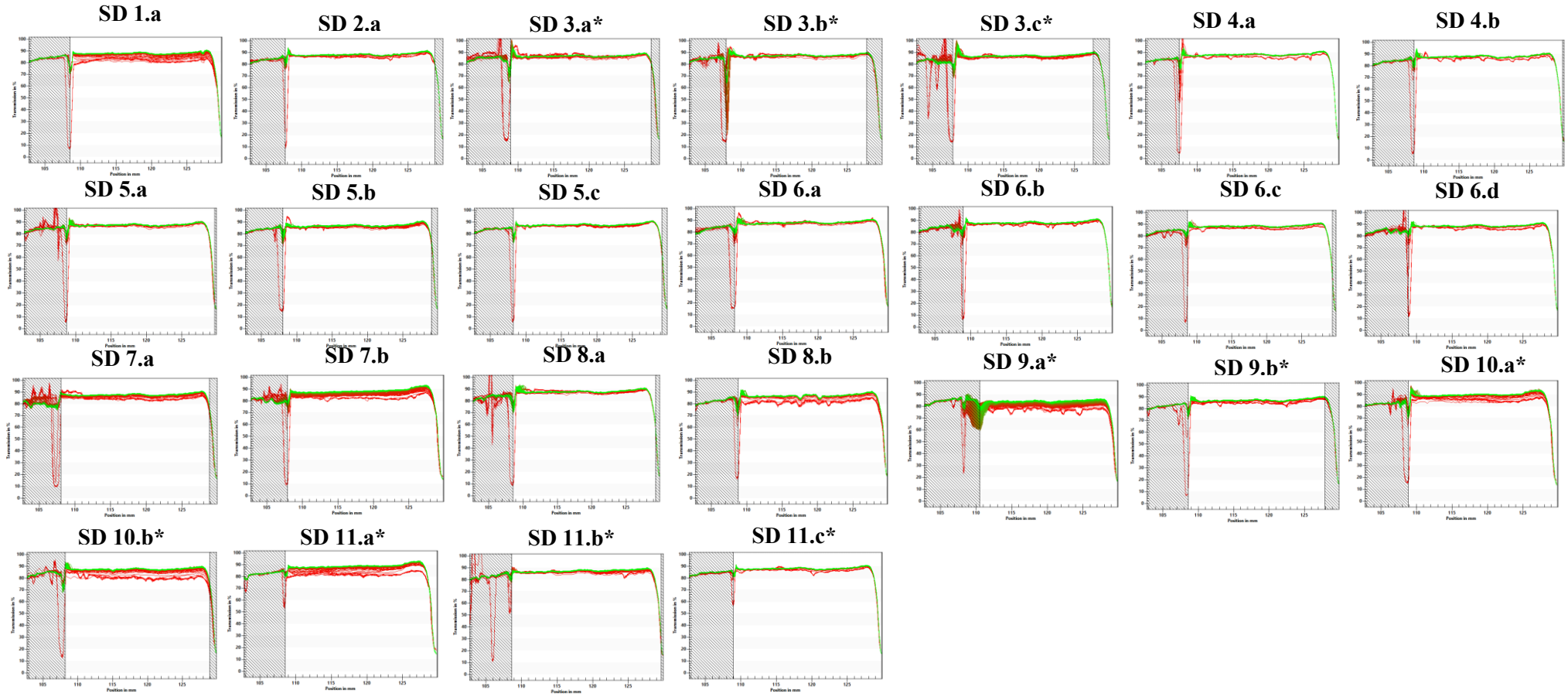
Online Retailers			
Amazon	GNC	Muscle Blaze	Team Ready
Beauty box Korea	Gymgrossisten	MyVegan	The Isopure Company
Behard	Holland and Barrett	No Pain No Gain	TruSource
Bevnet	Interspar	Nutrition	Ubuy
Biotech	Longos	NXT Nutrition	Upbeat
BiPro	MaxiNutrition	Powerfood	Vieve
Bodiez	Mcgrocer	Premier Protein	Vital Proteins
British online	Migros	Protein2O	Vitamin UK
supermarket	Multipower	Protein pick and mix	Walmart
Drink Trimino	Muscle and Strength	Prozis	Wowhydrate
Gatorade		Reneva	

Supplementary Table 2.1: List of online retailers used during the market research analysis.

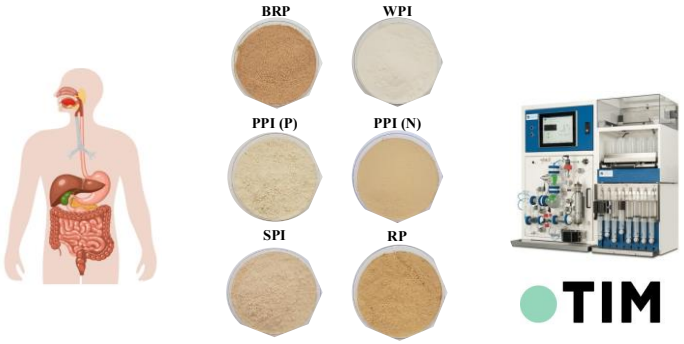
2.7. Supplementary Material



Supplementary Figure 2.1: Lumisizer graphs. Soft drink (SD), numbers 1-11 indicate product brand and the corresponding letter (a – d) shows different flavours of the same brand. An * - carbonated.

3. Chapter 3

Dynamic in-vitro system indicates good digestibility characteristics for novel upcycled plant protein; correlation to techno-functional properties



Published in Innovative Food Science and Emerging Technologies as: Jaeger, A., Ahern, N., Sahin, A. W., Nyhan, L., Mes, J. J., van der Aa, C., Vrasidas, I., & Arendt, E. K. (2024). Dynamic in-vitro system indicates good digestibility characteristics for novel upcycled plant protein; correlation to techno-functional properties. *Innovative Food Science & Emerging Technologies*, 103571. https://doi.org/10.1016/j.ifset.2024.103571

Abstract

An increasing global population demands the broadening of the plant-based protein portfolio. The high volume of by products produced across the food industry presents the opportunity to reduce food waste while reclaiming valuable nutrition. The current study examines a novel protein, barley and rice protein (BRP), extracted from brewers spent grains regarding its techno-functionality and digestibility, in comparison to a variety of proteins including whey, soy, rice and two pea protein sources. Compositional, foaming, emulsifying, structural and rheological properties were examined, while digestion kinetics were determined using the dynamic tiny TIM *in vitro* digestion model. Barley and rice protein performed similarly to whey protein in many respects, demonstrating a high solubility, high nitrogen bioavailability (>90%) and comparable nitrogen digestion kinetics, however BRP exhibited no rheological changes over heating. The digestible indispensable amino acid score (DIAAS) for BRP was 67% with lysine as the sole limiting amino acid, a higher value than soy, rice and one of the pea proteins investigated. The production of a protein ingredient from a plentiful and otherwise low value food processing by product presents an opportunity for an increased shift towards more sustainable protein sources for the food industry. This protein displays enhanced nutritional characteristics when compared to variety of plant protein sources, and promising functional properties such as a high solubility. This study offers an examination of a novel, upcycled protein ingredient as a potential tool for food manufacturers in the shift towards a more sustainable and secure food future.

3.1. Introduction

A growing global population, against the background of an increasingly unstable food system, presents a considerable challenge for the food industry. As of 2022, progress towards eliminating world hunger and malnutrition has been limited, with ~ 30 % of the global population experiencing food insecurity (FAO et al., 2022). In addition to this, a growing population with finite resources demands the rethinking of our current food systems. It is evident that the shift towards a more sustainable food system is mandatory, and that this must occur sooner rather than later. One way in which this issue can be addressed is through the increased revalorisation of food processing side streams (García-Oliveira et al., 2022). The brewing industry in particular produces large volumes of by products, namely spent grain, spent yeast and rootlets (Jaeger et al., 2020, 2021; Neylon et al., 2020). These by products have a high nutritional value, particularly with regards to protein and fibre, with recent studies highlighting the potential of these ingredients in bakery applications (Neylon et al., 2021; Neylon., et al., 2023). Further to this, the production of commonly used protein isolates also creates significant volumes of side streams such as starches and fibres, each presenting their own challenges with regards to incorporation into human nutrition. Therefore, the valorisation of existing food processing by products may be one way to tackle the move towards more sustainable food systems and increase the variety of food ingredients available to manufacturers.

Functionality is a major factor in determining the success of a commercial ingredient. Generally, food producers will favour a low cost material that performs according to the needs for specific applications e.g. protein solubility, oil and water binding, emulsion stabilisation and sedimentation behaviour among others all give a practical overview of each protein ingredient and its potential use in product formulation. As the protein transition should not compromise human health, an exploration of protein quality is also essential. It is well known that many plant based protein sources do not reach the recommended level of all essential amino acids, in particular sulphur containing amino acids in legumes and lysine in cereal based proteins. It has also been shown that plant based proteins are generally less digestible than their animal based counterparts, due to variety of factors including the increased presence of antinutritional compounds. Protein quality can be assessed using a parameter such as the digestible indispensable amino acid score, or DIAAS, where the true bioavailability of amino acids is compared to a reference standard as defined by (FAO, 2013). Protein bioavailability is

ideally determined using *in-vivo* trials in human or animal models, however such studies are invasive, expensive, and require ethical approval. *In vitro* systems, such as the dynamic digestion system called the Tiny TIM (TNO Gastro- Intestinal Model), used in the current study, offer accurate and reliable alternatives to animal and human studies (Kleiveland et al., 2015).

The current study examines a novel food ingredient isolated from brewers spent grain (BSG), comparing it to a variety of protein ingredients currently widely utilised in food manufacturing with regards to protein digestibility and amino acid bio-accessibility while also exploring protein functionality. While studies on protein ingredients of this kind have been limited, particularly regarding digestibility characteristics, recently published data shows that spent grain protein exhibits comparable post-prandial total amino acid uptake to pea protein, with a lower lysine and higher tryptophan level respectively (Ummels et al., 2023). The aim of this study is to provide an overview of how a novel protein isolate may be used in the food industry in place of, or in addition to protein ingredients which are already widely used, providing food manufacturers more opportunity to shift towards a more sustainable food future.

3.2. Materials and methods

3.2.1. Materials

The protein isolates used in this study were sourced commercially (**Table 3.1**).

Table 3.1: Summary of commercial protein isolates.

Ingredient Description	Abbreviation	Source
Everpro®- Spent grain (barley-rice) protein isolate	BRP	EverGrain Ingredients, MO, USA
Pisane C9- Pea protein isolate	PPI (P)	Cosucra, Warcoing, Belgium
Provon 190 – Whey Protein Isolate	WPI	Glanbia Nutritionals, Kilkenny, Ireland
Supro 661 G – Soy Protein Isolate	SPI	International Flavours and Fragrances, NY, USA
Brown Rice Protein Isolate	RP	Axiom Foods, CA, USA
Nutralys S85 Plus N – Pea protein isolate	PPI (N)	Roquette Freres, Lestrem, France

3.2.2. Compositional analysis

Compositional analysis was carried out externally by Chelab S.r.l. (Resana, Italy), using the following methods: Moisture, fat, sugars and ash were quantified using the internal methods based on AOAC and ISO standards; total starch was determined using the Megazyme kit K-RAPRS (Megazyme, Bray, Ireland); The amino acid composition was determined externally by Chelab S.r.l. (Resana, Italy) using ion chromatography with post-column derivatization with ninhydrin, or HPLC-UV analysis in the case of tryptophan. Protein content was taken as the sum of individual anhydrous amino acid residues.

3.2.3. Calculation of conversion factors

The conversion factor K_p was calculated as defined by Mossé (1990) using the following equation:

$$kp = \frac{\Sigma Ei}{N}$$

Where E_i is the sum of anhydrous amino acid residues and N is the total nitrogen value as determined by the Kjeldahl method.

3.2.4. Techno-functional properties

3.2.4.1. pH and total titratable acidity (TTA)

The pH and TTA of each ingredient as dissolved in water was determined using a pH meter (Mettler Toledo, Ohio, United States) as described by Jaeger et al. (2023) and the results are presented as ml of 0.1 M NaOH/g of sample.

3.2.4.2. Foaming capacity and foam stability

Foaming properties of samples at an aqueous concentration of 2 % w/w powder were examined as described in Alonso-Miravalles et al. (2019). Briefly, samples were frothed using an Ultraturrax T10 (IKA Labortechnik, Germany) with S10N-10G dispersing element, at maximum speed for 30 s and the height of the foam phase was measured. Sample expansion was calculated using the following equations:

$$Foaming\ capacity\ (\%) = \left(\frac{Foam\ height\ immediately\ after\ foaming}{Initial\ sample\ height}\right) \times 100$$

$$Foam\ stability\ (\%) = \left(\frac{Foam\ height\ after\ 1\ hour}{Foam\ height\ immediately\ after\ foaming}\right) \times 100$$

3.2.4.3. Protein Profile Analysis

The protein profile of all samples was determined by SDS – PAGE under reducing conditions using the NuPAGE Bis- Tris mini gels (4 -12 % 1mm 12 well) (Invitrogen, Thermo Fisher Scientific, CA, USA). Protein was extracted at 2% (w/w) from each sample using an extraction buffer consisting of 5 M Urea, 2 M Thiourea, 1 mM EDTA, 2% SDS and 0.1 M Tris base, adjusted to pH 8.8 using 3 M HCL, using 1.75% 2-Mercaptoethanol (BME). Samples were shaken at 500 rpm for 16 h at room temperature followed by centrifugation at 14,680 rpm for 20 min after which an aliquot was mixed with sample buffer and heated at 70°C for 10 min. Precision plus Protein^{tm} dual xtra Prestained standard (BioRad, CA, USA) ladder with a size range of 2 – 250 kDa was prepared. 20 µl of diluted ladder and sample were loaded into the gel and ran for 35 min at 200 V constant. Following the completion of electrophoresis, gels were rinsed and fixed (40% methanol, 10% acetic acid) for 30 min while shaking, stained (0.025% Coomassie G250) for 20 min and destained (10% acetic acid) until background colour was removed.

3.2.4.4. Minimum gelation concentration

Minimum gelation concentration of each protein was determined according to the method of Alonso-Miravalles et al. (2019), with some modifications as described by Jaeger et al. (2023). Briefly, samples (15 ml) were prepared at a variety of concentrations (in the range of 6% to 30%, in 2% intervals). The samples were then adjusted to pH 7 using varying concentrations (0.01 M–2 M) of HCl and NaOH, and hydrated overnight at 4°C. Samples were heated at 90°C in a water bath for 30 min, cooled rapidly on ice, and maintained overnight at 4°C. The samples were then inverted, and the minimum protein concentration at which the dispersion did not flow in less than 30 s was determined as the minimum gelling concentration

3.2.4.5. Rheological characteristics

Rheological tests were carried out using a controlled stress rheometer (MCR301, Anton Paar GmbH, Austria) equipped with a concentric cylinder measuring system (C-CC27-T200/SS, Anton Paar GmbH, Austria) according to the method of Vogelsang-O'Dwyer et al. (2020). Sample concentrations were decided based on the minimum gelling concentrations of each ingredient i.e. PPI (P): 12%, RP: 18%, SPI: 14%, WPI: 10% and PPI(N) 26%. Since no minimum gelling concentrations were determined for BRP, BRP was tested at the minimum gelling concentrations for WPI (10%) and PPI (N) (26%).

3.2.4.6. Sulfhydryl groups

Exposed, free and total sulfhydryl (SH) groups were determined using Ellman's reagent [5,5′-dithio-bis-(2-nitrobenzoic acid)] according to the method of Alonso-Miravalles et al. (2019) with some adjustments. Protein samples of 75 mg were extracted overnight at 4°C in 10 ml Tris-Glycine buffer at pH 8 consisting of 0.1 M Tris, 0.1 M glycine and 4 mM EDTA for exposed SH groups with the addition of 8 M Urea to Tris-Glycine buffer for free and total SH groups. To determine exposed and free SH groups, 80 µl of Ellman's reagent was added to 2.5 ml of diluted sample, including a reagent blank. Sample blanks were prepared by replacing Ellman's reagent with the respective Tris-Glycine buffer. Samples were developed at room temperature for 15 min, centrifuged at 4000 rpm for 15 min at 22°C and absorbance was measured on a spectrophotometer at 412 nm. For total SH groups, 4 ml Tris-Glycine buffer with urea and 50 µl BME was added to 1 ml diluted protein sample and shaken at 500 rpm at room temperature for one hour. This was followed by the addition of 10 ml 12% trichloroacetic acid (TCA) to the sample solutions and a one hour shaking period. To remove the BME the supernatant was discarded, and the pellet resuspended twice in 12% TCA followed by

centrifugation at 4893 *x g* for 15 min at 22 °C. The pellet was resuspended in 10 ml Tris-Glycine buffer containing 8 M urea and total SH content was determined following the same procedure as exposed and free SH groups, instead using half the volume of Ellman's reagent and double the volume of diluted sample with no centrifugation step before reading the absorbance. The content of SH groups was calculated as follows:

$$\mu M \frac{SH}{g} protein = \frac{((A\,412 - A\,412\,a - A\,412\,b) * 1000000 / \varepsilon)}{C}$$

Where A_{412} is the absorbance at 412 nm, A_{412a} and A_{412b} are the absorbance values for the blank and reagent blank, respectively, ε is the extinction coefficient which was taken as 13,600 M^{-1} cm^{-1} and C is the protein concentration in mg/mL of the diluted sample.

3.2.4.7. Surface Hydrophobicity

Surface hydrophobicity was measuring using 1-anilinonaphthalene-8-sulphonate as a fluorescent marker, as described in literature (Li et al., 2018; Vogelsang-O'Dwyer et al., 2022). Sample dispersions were prepared and analysed according to the method described by Alonso-Miravalles et al. (2019). Briefly, protein dispersions were serially diluted with 10 mM phosphate buffer (pH 7) in the range of 0.0006–0.015% (w/v). Then, 8-Anilino-1-naphthalenesulfonic acid ammonium salt (ANS) (10 µl; 8.0 mM in 0.1 M phosphate buffer, pH 7) was mixed with 2 ml of diluted sample and stored in the dark for 15 min at room temperature. Fluorescence was measured (λ excitation 390 nm, λ emission 470 nm) and corrected using blanks, sample dilutions measured without the addition of ANS. The results are presented as the slopes ($R2 \geq 0.98$) of the absorbance versus protein concentration (% w/v).

3.2.4.8. Water and Oil holding capacity

Water holding (WHC) and oil holding (OHC) capacity, was determined using the method described by Boye et al. (2010), with slight modifications. Briefly, 1 g of ingredient was mixed with 6 g of either deionised water or sunflower oil. The sample mixtures were allowed stand for 1 h for sufficient uptake of water or oil following centrifugation at 4000 rcf at 20 °C for 30 min. The supernatant was then carefully removed before being inverted for 30 min to allow sufficient drainage. WHC/OHC (%) was calculated using the following formula:

$$\frac{WHC}{OHC}(\%) = \frac{(Weight\ of\ tube+pellet)-(Weight\ of\ empty\ tube)}{Weight\ of\ ingredient\ input} \times 100\ \%$$

3.2.4.9. Protein Solubility

Protein solubility was determined at various pH values according to Alonso-Miravalles et al. (2019) with modifications. Sample dispersions of 1% (w/w) protein were prepared in distilled water followed by pH adjustment to pH 3, 5 or 7 using varying concentrations (0.01 – 1 M) HCl and NaOH. The dispersions were shaken at 500 rpm overnight at 4°C. The pH was readjusted if necessary and then centrifuged at 4000 rpm for 30 min at 20°C. The protein content in the supernatant was measured using the Kjeldahl method AACC Method 46-12 (AACC International, 2011) and calculated based on nitrogen to protein Kp conversion factors for each individual ingredient. Protein solubility was expressed as the % of protein remaining in the supernatant.

3.2.4.10. Emulsifying Characteristics

Emulsion stability was measured as described by Vogelsang-O'Dwyer et al. (2021) using an analytical centrifuge (Lumisizer ,GmbH, Berlin, Germany), with parameters as defined by Jaeger et al. (2023). Emulsions of 1% (w/v) protein were prepared with ultrapure water with the addition of 10% sunflower oil. Results are given as separation rate (%/min) of individual emulsions and light transmission profiles.

3.2.4.11. Colour

The Commission Internationale de L'Eclairage (XYZ) values were measured to obtain the Hunter colour system CIE (L* a* b*) values of each ingredient as described by Jaeger et al. (2023)**.** The whiteness index (WI) was calculated according to the following equation (Boeck et al., 2022):

$$WI = 100 - \sqrt{((100 - L*)^2 + a*^2 + b*^2)}$$

3.2.4.12. Ultrastructure

Scanning electron microscopy (SEM) was used to examine the ultrastructure of all samples according to the method reported by Atzler et al. (2021). The following settings were applied for the analysis: 5 kV voltage, 20 mm working distance and a magnification factor of 1000x.

3.2.5. Digestion kinetics

3.2.5.13. In-vitro digestion simulation

Adult gastrointestinal conditions were simulated using the tiny-TIM model (Havenaar et al., 2013, 2016) an *in-vitro* model with the ability to assess true ileal digestibility as well as the

bio-accessibility of nitrogen and amino acids. This was completed externally by The TIM Company and their analytical lab partners Ducares-Triskelion and BioAnalytiX.

The tiny-TIM model consists of a gastric compartment and one small intestinal compartment connected by a peristaltic valve (pyloric sphincter). It simulates very closely the successive dynamic conditions in the upper gastrointestinal tract, such as body temperature, pH curves, concentrations of electrolytes, digestive enzymes. The enzymes and bile used were sourced from Sigma-Aldrich NV (Zwijndrecht, The Netherlands). Specifically, α-amylase from *Bacillus* sp, pepsin from porcine gastric mucosa, lipase from *Rhizopus oryzae*, and porcine bile were utilised in this study. The set-points for gastrointestinal simulation were controlled and monitored by specific computer programs. During the experiments, the digested and dissolved molecules were dialysed (diffused out) from the intestinal lumen through a semipermeable membrane unit (Fresenius FX-5 dialyser, Fresenius Medical Care, Bad Homburg, Germany) with a cutoff of 5-7 kDa connected to the small-intestinal compartment. This allowed sampling during the digestion process and the assessment of the bioaccessible fraction in the dialysate. The dialysate represents the bioaccessible fraction of the digestate, determined by the ability of the nutrients to pass from the lumen by a semi-permeable membrane unit.

Total gastric intake was 150 g, with 7 g of sample per run in combination with water, simulated saliva and gastric start residue. For the blank experiments, 15 g of citrate buffer was included to replace the sample. The conditions simulated were the standard post-meal conditions of an average, healthy human adult (**Table 3.2**). A total of 10 samples were collected per tiny-TIM run: 7 dialysate samples collected in 15, 30, and 60 minute aliquots (i.e. at 0-15 min, 15-30 min, 30-60 min, 60-120 min, 120-180 min, 180-240 min, 240-300 min), 1 gastric residue (including rinse) sample, 1 intestinal residue (including rinse), and a pooled dialysate sample (0-300 min). The digestion of each sample and the blank was performed in duplicate, resulting in 14 individual tiny-TIM runs.

Table 3.2: Overview of parameters utilised in the TinyTIM in-vitro gastrointestinal model.

Stomach	
Intake (total)	150 grams
Gastric half emptying time (t1/2)	50 minutes
Gastric pH	6.5 to 2.0 in 90 minutes
Enzymes	Amylase, lipase, pepsin
Small Intestine	
Small intestinal pH	6.5
Bile Secretion	
Pancreatic enzymes secretion	Containing trypsin, amylase, lipase, protease
Temperature	37 ± 1 °C
Experiment duration	5 hours

3.2.5.14. Analysis of dialysate samples

Each of the collected samples, as well as the initial isolates, were analysed for total nitrogen concentration using the Kjeldahl method (ISO 5983-2). The initial protein isolates and the final pooled dialysate (0-300 min) were analysed for their content of 9 essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) and 2 conditionally essential amino acids (tyrosine and cysteine) using LC-MS/MS, and UPLC in the case of tryptophan, for calculation of digestible indispensable amino acid score (DIAAS). This was performed after hydrolysis with 6 M HCl, and $Ba(OH)_2$ in the case of tryptophan. For the determination of cystine and methionine the procedure described is proceeded by oxidation with performic acid for 16 – 20 hours. Alpha-amino nitrogen was determined by UV spectroscopy at 405 nm, using 2,4,6- trinitrobenzen-sulphonic acid (TNBS) as a derivatising agent.

True ileal digestibility is calculated by correcting the absolute amount of nitrogen or individual amino acid recovered from the TIM samples for the absolute amount of the blank samples, relative to the exogenous input:

$$True\ ileal\ digestibility\ (\%) = \frac{\sum A\ dialysate - \sum A\ blank}{A\ exogenous\ input}.100$$

The digestible indispensable amino acid (DIAA) reference ratio of the essential amino acids was calculated using the sum of each amino acid content in the pooled fraction (0-300 min), and the digestible indispensable amino acid score (DIAAS) was calculated from the lowest DIAA, according to the adult essential amino acid reference patterns (FAO, 2013).

$$DIAAS\ (\%) = \frac{limiting\ DIAA\ [mg]\ in\ 1\ g\ test\ protein}{limiting\ DIAA\ [mg] in\ 1\ g\ reference\ protein}\ .100$$

3.2.6. Statistical analysis

All analyses were completed in triplicate unless stated otherwise. Results were tested for normality and a one-way ANOVA with post hoc Tukey test ($p < 0.05$) was performed using IBM SPSS version 26 (Armonk, NY, USA). When equal variances were not assumed, a correction using the Welch test and Games Howell post hoc test ($p < 0.05$) was applied. When data were not normally distributed a non-parametric Kruskal Wallis test ($p < 0.05$) was performed. Principal component analysis (PCA) was performed using Origin Pro 2023b (MA, USA), where surface hydrophobicity was excluded due to missing values.

3.3. Results

3.3.1. Compositional Analysis

The compositional data of the protein isolates examined in this study are displayed in **Table 3.3**. BRP contained a high level of protein (79.29 g/100 g) when compared to the other plant-based samples (69.87 – 71.42 g/100 g), second only to WPI protein (90.42 g/ 100 g) and similar to SPI (76.19 g/100 g). BRP and WPI contained only trace levels of fat, while both of the pea proteins tested contained a higher amount with 8 – 9%. Examining carbohydrate composition, all samples contained minimal amounts of starch, with the exception of RP, although levels were still low. BRP contained a small amount of glucose and traces of fructose. RP and PPI (N) also contained glucose, but at lower levels. The sugar present in the highest amount throughout all the ingredients was sucrose in RP. Sucrose was also present in PPI (P), SPI, and PPI (N) but at significantly lower levels. The primary sugar present in WPI was lactose, with none of the other screened sugars detected. Maltose was only present in small amounts in RP and PPI (N). Amongst the six samples, BRP, PPI (P) and PPI (N) contained similar levels of ash (~5%), while the other samples contained 2.5 – 3 %. All ingredients contained similar levels of sodium (1 – 1.5 %) with the exception of WPI protein with a lower level (0.29 g/100 g).

Table 3.3: Compositional analysis of all protein isolates, expressed as g/100 g as mean ± standard deviation on "as-is" and dry matter basis. <LoQ indicates that the obtained result was below level of quantification for the method of analysis, *Protein value calculated using individual amino acid residues; relative standard deviations (RSD) <10%, <20% in the case of tryptophan, methionine and cysteine.

	BRP		**PPI (P)**		**RP**		**SPI**		**WPI**		**PPI (N)**	
	g/100g as-is	**g/100g DM**	**g/100g as-is**	**g/100g DM**	**g/100g as-is**	**g/100g DM**	**g/100g as-is**	**g/100g DM**	**g/100g as-is**	**g/100g DM**	**g/100g as-is**	**g/100g DM**
Moisture	7.23 ± 0.26		5.19 ± 0.19		3.81 ± 0.14		5.21 ± 0.19		3.80 ± 0.14		7.26 ± 0.26	
Protein	79.29 ± 2.45	85.47 ± 2.64	69.87 ± 2.07	73.69 ± 2.18	71.42 ± 2.03	74.25 ± 2.11	76.19 ± 2.23	80.38 ± 2.35	90.42 ± 2.71	93.99 ± 2.82	69.63 ± 2.07	75.08 ± 2.23
K_P	6.19		5.78		5.85		5.82		6.65		5.93	
Fat	0.07 ± 0.03	0.08 ± 0.04	8.92 ± 0.54	9.41 ± 0.57	1.95 ± 0.12	2.03 ± 0.13	3.47 ± 0.21	3.66 ± 0.22	<LoQ	<LoQ	7.99 ± 0.48	8.62 ± 0.52
Carbohydrate												
Starch	<LoQ	<LoQ	<LoQ	<LoQ	3.98 ± 0.11	4.13 ± 0.11	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ
Sugars												
Glucose	0.436 ± 0.033	0.470 ± 0.036	<LoQ	<LoQ	0.143 ± 0.013	0.149 ± 0.014	<LoQ	<LoQ	<LoQ	<LoQ	0.030 ± 0.007	0.032 ± 0.008
Fructose	0.088 ± 0.009	0.095 ± 0.009	<LoQ	<LoQ	0.045 ± 0.007	0.046 ± 0.008	<LoQ	<LoQ	<LoQ	<LoQ	0.084 ± 0.009	0.091 ± 0.009
Lactose	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	0.247 ± 0.033	0.257 ± 0.034	<LoQ	
Sucrose	<LoQ	<LoQ	0.270 ± 0.027	0.285 ± 0.028	4.900 ± 0.480	5.094 ± 0.499	0.214 ± 0.022	0.226 ± 0.023	<LoQ	<LoQ	0.192 ± 0.020	0.207 ± 0.022
Maltose	<LoQ	<LoQ	<LoQ	<LoQ	0.202 ±0.031	0.210 ± 0.032	<LoQ	<LoQ	<LoQ	<LoQ	0.123 ± 0.020	0.133 ± 0.022
Ash	5.04 ± 0.34	5.43 ± 0.37	5.00 ± 0.34	5.27 ± 0.36	2.76 ± 0.19	2.87 ± 0.20	3.46 ± 0.23	3.65 ± 0.24	2.68 ± 0.18	2.80 ± 0.19	4.94 ± 0.33	5.33 ± 0.36
Sodium	1.31 ± 0.24	1.41 ± 0.26	1.58 ± 0.29	1.67 ± 0.31	1.06 ± 0.20	1.10 ± 0.21	1.02 ± 0.19	1.08 ± 0.20	0.29 ± 0.05	0.31 ± 0.06	1.30 ± 0.24	1.40 ± 0.26

3.3.2. Physical and Techno-functional Properties

Table 3.4: Functional and physical properties of protein isolates. Results are expressed as mean ± standard deviation. Results contained in the same row with the same letter marking do not differ significantly ($p < 0.05$).

	BRP	PPI (P)	RP	SPI	WPI	PPI (N)
pH	7.40 ± 0.01 [a]	7.50 ± 0.02 [b]	8.06 ± 0.01 [e]	6.83 ± 0.02 [d]	6.31 ± 0.02 [c]	7.08 ± 0.02 [f]
TTA (mL 0.1 M NaOH/g)	2.44 ± 0.03 [a]	0.81 ± 0.01 [b]	0.25 ± 0.01 [e]	1.74 ± 0.25 [d]	2.39 ± 0.01 [c]	2.12 ± 0.01 [f]
Foaming Capacity (%)	138.79 ± 3.06 [a]	36.13 ± 3.18 [b]	91.01 ± 7.79 [c]	41.21 ± 1.84 [b]	143.94 ± 3.47 [a]	40.15 ± 1.31 [b]
Foaming Stability (%)	44.60 ± 1.10 [a]	98.04 ± 3.40 [b]	38.83 ± 2.10 [a]	99.89 ± 5.72 [b]	41.08 ± 2.32 [a]	88.89 ± 9.62 [b]
Surface hydrophobicity (AU)	n/a	6816.33 ± 548 [a]	6040.86 ± 384 [a]	6434.33 ± 418 [a]	1516.33 ± 151 [b]	7951.00 ± 387 [c]
Minimum gelling concentration (%)	n/a	12	18	14	10	26
Water holding capacity (%)	n/a	394.16 ± 6.38 [a]	201.98 ± 2.05 [c]	310.67 ± 3.49 [b]	n/a	98.17 ± 1.42 [d]
Oil holding capacity (%)	236.65 ± 1.46 [a]	79.40 ± 7.80 [b]	86.98 ± 1.18 [bd]	84.83 ± 0.80 [bd]	23.20 ± 1.60 [c]	90.80 ± 2.37 [d]
Separation rate (%/min)	2.86 ± 0.11 [a]	0.97 ± 0.02 [b]	2.43 ± 0.25 [d]	1.82 ± 0.05 [c]	2.87 ± 0.11 [a]	0.90 ± 0.01 [e]
Whiteness index (%)	58.17 ± 0.92	67.26 ± 0.46	59.71 ± 1.16	77.75 ± 1.21	87.67 ± 1.00	56.81 ± 0.99
Exposed SH groups (µM/g protein)	0.41 ± 0.02 [a]	0.07 ± 0.05 [b]	1.69 ± 0.03 [e]	3.63 ± 0.13 [d]	16.60 ± 0.02 [c]	11.14 ± 0.13 [f]
Free SH groups (µM/g protein)	0.17 ± 0.01[a]	0.67 ± 0.12 [a]	1.25 ± 0.09 [c]	3.47 ± 0.58 [c]	21.66 ± 0.21 [b]	10.93 ± 0.25 [d]
Total SH groups (µM/g protein)	4.17 ± 1.04 [a]	76.29 ± 1.49 [be]	81.37 ± 4.00 [e]	71.29 ± 2.59 [bd]	93.15 ± 2.10 [c]	66.26 ± 4.79 [d]
Protein solubility at pH 3 (%)	91.05 ± 4.51 [a]	13.05 ± 0.75 [b]	11.88 ± 0.25 [b]	23.58 ± 1.15 [c]	94.73 ± 1.25 [a]	29.22 ± 0.75 [d]
Protein solubility at pH 5 (%)	91.47 ± 0.50 [a]	4.56 ± 0.08 [b]	8.63 ± 0.50 [c]	4.62 ± 0.10 [b]	86.16 ± 1.97 [a]	27.57 ± 1.28 [d]
Protein solubility at pH 7 (%)	95.16 ± 1.84 [a]	30.64 ± 1.37 [b]	19.33 ± 0.99 [c]	30.89 ± 0.94 [b]	97.82 ± 0.45 [a]	44.89 ± 0.76 [d]

3.3.2.15. pH and Total Titratable Acidity (TTA)

The pH and TTA are presented in **Table 3.4**. All pH values are within the range of 6.31 – 8.06 with WPI and RP exhibiting the lowest and highest pH values, respectively. BRP and WPI displayed similar TTA values at 2.44 ml and 2.39 ml of 0.1M NaOH/g. RP presented the lowest TTA result at 0.25 ml 0.1M NaOH/g, followed by PPI (P), SPI and PPI (N) respectively.

3.3.2.16. Foaming Properties.

Foaming capacity and foam stability of all the protein ingredients are presented in **Table 3.4**. WPI and BRP displayed high foaming capacities (139% and 144%, respectively), and low foam stabilities of 41% and 45%, respectively. Both pea derived proteins, PPI (P) and PPI (N), showed similar foaming behaviour with low foaming capacities (33.57 - 34.14%) and relatively high foam stabilities (97.91 - 98.04%). SPI also follows this trend with a foaming capacity of 41% and a stability of 99%. RP falls between these two extremes with a foaming capacity of 91% but possesses the lowest foam stability of 39%.

3.3.2.17. Surface hydrophobicity

The surface hydrophobicity of the samples is also presented in **Table 3.4**. PPI (P), RP and SPI exhibited similar results (6040 - 6816 A.U.) whereas PPI (N) had the highest hydrophobicity of all ingredients with a value of 7951 A.U. In contrast to this, WPI exhibited the lowest hydrophobicity with a value of just 1516 A.U. It was not possible to determine a result for BRP.

3.3.2.18. Water and Oil Holding capacity

As shown in **Table 3.4**, water holding capacity could not be determine for BRP and WPI due to their high solubility in water. PPI (P) showed the highest WHC at 394.16% followed by SPI, RP and PPI (N) at 310.67%, 201.98% and 98.17%, respectively. BRP had a significantly higher OHC than the other samples at 236.65%. PPI (N), RP, SPI and PPI (P) had OHC values of 90.8%, 86.98%, 84.83% and 79.40%, respectively, while WPI showed the lowest ability to absorb oil at 23.20%.

3.3.2.19. Emulsifying characteristics

The separation rates of all emulsion dispersions are shown in **Table 3.4.** WPI emulsion exhibited a separation rate of 2.87 %/min, whereas PPI (N) emulsion separated more slowly at 0.9 %/min. BRP emulsion also separated quickly with a separation rate of 2.86 %/min, similarly to WPI. SPI and RP emulsions showed a separation rate of 1.82 and 2.43 %/min,

respectively. PPI (P) and PPI (N) both showed lower separation rates, indicating better emulsifying ability. **Figure 3.1** displays the light transmission curve of each sample, alongside sample cuvettes. Little to no sedimentation was observable in BRP (**Figure 3.1A**) and WPI (**Figure 3.1E**) while significant sedimentation can be seen in PPI (P) (**Figure 3.1B**), RP (**Figure 3.1C**), SPI (**Figure 3.1D**) and PPI (N) (**Figure 3.1F**).

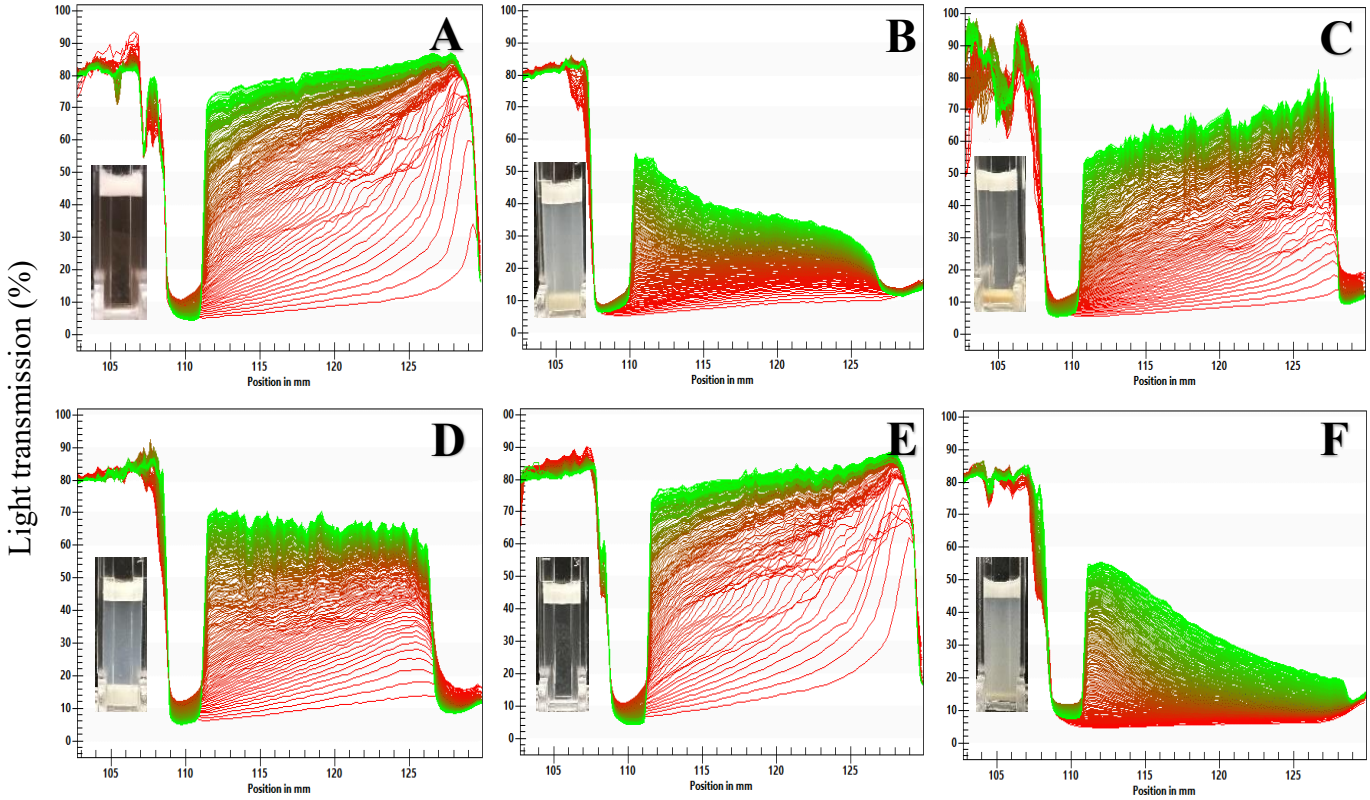


Figure 3.1: Light transmission profiles of (A) BRP, (B) PPI (P), (C) RP, (D) SPI, (E) WPI and (F) PPI (N) emulsion as a function of time.

3.3.2.20. Colour

Whiteness index (WI) of the ingredients is shown in **Table 3.4**. WPI displayed the highest WI (87.7 ± 1) whereas PPI (N) had the lowest (56.8 ± 1). A pure white colour is represented by 100%. The whiteness index of the other ingredients was within the range of 56.81 – 87.67%.

3.3.2.21. Sulfhydryl groups

Exposed, free and total SH groups are shown in **Table 3.4**. WPI exhibited the highest concentration of exposed, free, and total SH groups at 16.60 µM/g, 21.66 µM/g, and 93.15 µM/g of protein, respectively. BRP obtained the lowest concentration of free SH groups at 0.17 µM/g and total SH groups at 4.17 µM/g protein, while PPI (P) showed the lowest concentration of exposed at 0.07 µM/g. After WPI, total SH groups were found to be higher in RP (81.37

µM/g), followed by PPI (P) (76.29 µM/g) and SPI (71.29 µM/g) which gave similar values, and lastly PPI (N) at 66.26 µM/g protein, respectively.

3.3.2.22. Protein solubility

Figure 3.2 represents the protein solubility of all ingredients at pH 3, pH 5 and pH 7. BRP and WPI displayed consistently high solubility values across the tested pH range. At pH 7, WPI demonstrated the highest solubility (97.82%), followed by BRP with 95.16%. The lowest solubility for all ingredients was found at pH 5 with the exception of BRP at pH 3. PPI (P) and SPI showed similar results at pH 5, with solubility values of 4.56% and 4.62%, respectively.

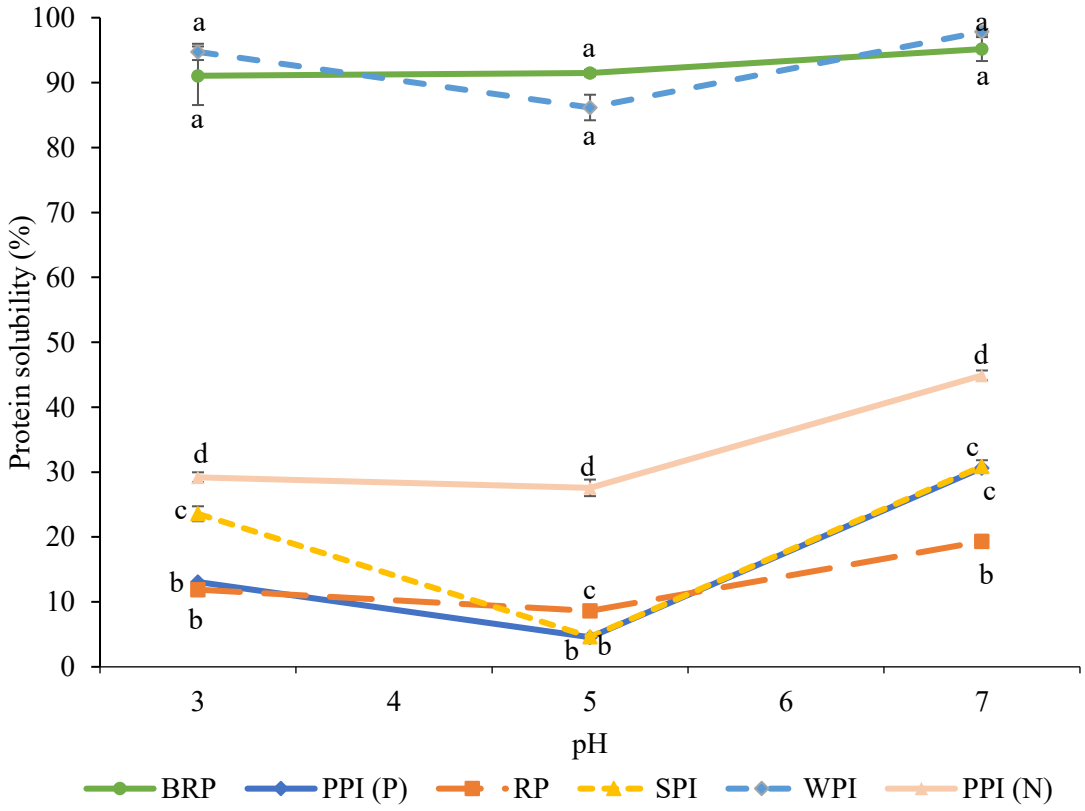


Figure 3.2: Protein solubility of protein ingredients at pH 3, pH 5, and pH 7, expressed as % protein in supernatant over initial protein concentration.

3.3.2.23. Protein profile

Figure 3.3 shows the electrophoretogram of all protein samples. Minimal bands are present in BRP, with the exception of a faint band at ~ 37 kDa. However, there is an increase in density of small peptides towards the lower molecular weights (<10 kDa), suggesting extensive protein hydrolysis, indicated by a deeper blue colour in this region. Both pea protein isolates exhibit similar band profiles, with some evidence of hydrolysis apparent in PPI (N) with increased

density at lower molecular weights. Several bands present in PPI (P) are either less defined or absent in PPI (N). Similarly to PPI (P) and PPI (N), SPI displays significant bands at ~70 kDa, ~ 30 kDa and ~ 20 kDa. However, there is also a smaller band present at ~12 kDa. RP shows two significant bands at ~12 – 15 kDa while WPI exhibits well resolved bands at ~75 kDa and ~60 kDa, as well as bands between 15 and 20 kDa and at ~14 kDa. Significant protein aggregation is also visible in PPI (P), RP and SPI, with a lesser degree in PPI (N), as an accumulation in the gel well.

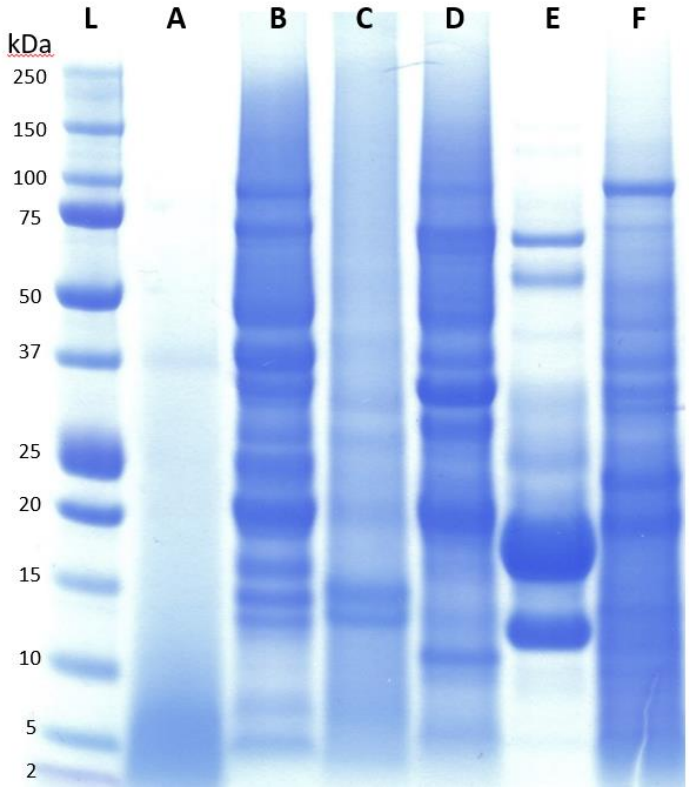


Figure 3.3: SDS-PAGE electrophoretogram under reducing conditions of BRP (A), PPI (P) (B), RP (C), SPI (D), WPI (E), and PPI (N) (F) alongside a molecular weight marker (L). Distinct polypeptide bands of the protein subunit composition present in each isolate.

3.3.2.24. Viscoelastic behaviour

Figure 3.4 shows the rheological behaviour of the protein isolates over a heating and cooling cycle. G', the storage modulus, represents the elastic (solid-like) response of a material while G'', the loss modulus, represents the viscous (or liquid-like) response. BRP displayed unmeasurably low G' and G'' values without change over the testing period. In pretrials, BRP was tested for gelling behaviour at concentrations up to 30% w/w. PPI (P) and SPI displayed similar behaviour over the cycle, with G' dominating over the length of the profile. Starting with a relatively high G', this then decreased significantly during heating followed by an increase upon cooling. RP followed a different pattern, with G' slowly increasing during heating, and further strengthening during cooling. WPI displayed the strongest gel forming

behaviour, with G' gradually increasing during heating with a notable increase at the initiation of cooling. The crossover point of a material is described as the point at which G' crosses over G'', indicating a dominance of elastic behaviour, also called "gel-point" (Shevkani et al., 2019). For WPI, this occurred at 32 min, when the sample had reached 83.5 °C. PPI (N) displayed vastly different rheological behaviour to PPI (P), under the applied conditions. In contrast, G'' initially dominated, with the gel point occurring at 16 min, at a temperature of 47.5 ° C. In this instance, PPI (N) had a profile most similar to that of WPI, with a gradual increase in G' during heating with a significant strengthening during cooling to give similar final G' values.

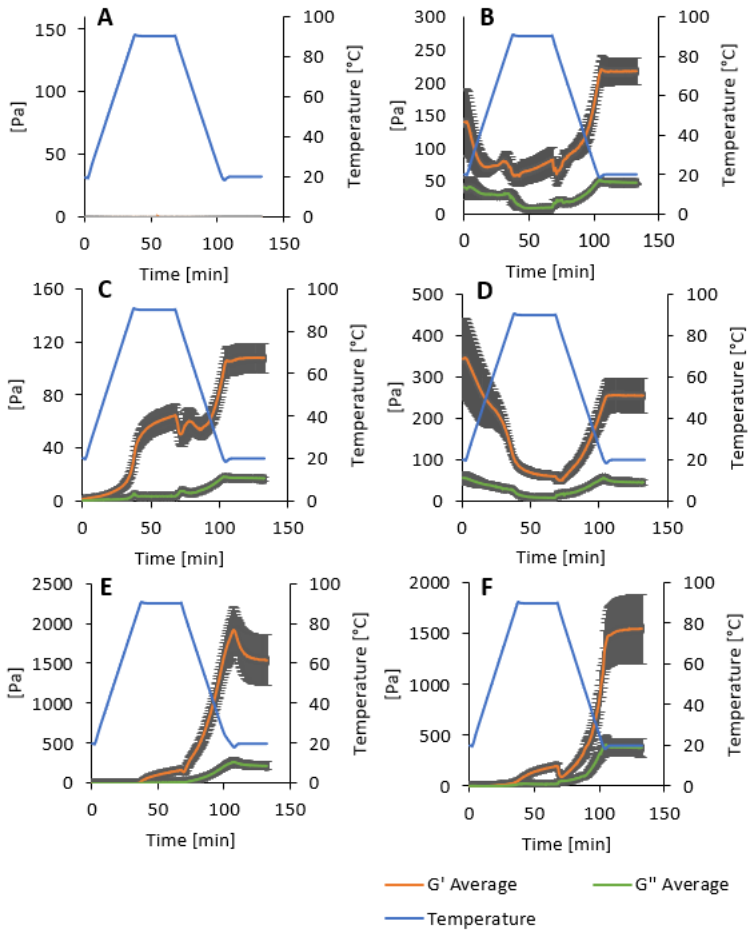
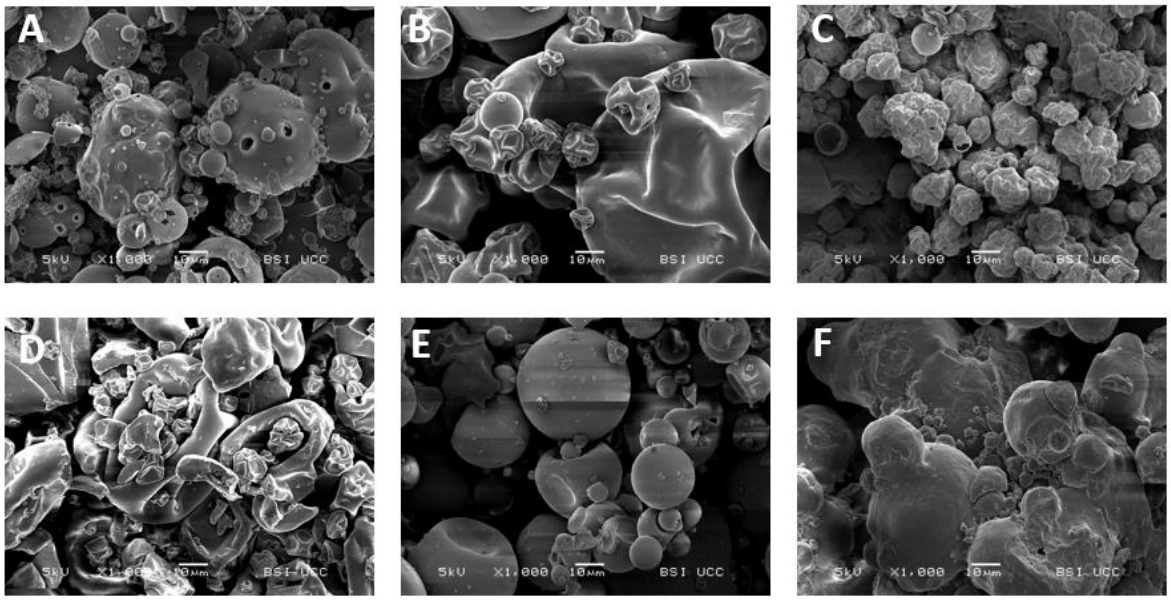


Figure 3.4: Rheological behaviour of BRP (A), PPI (P) (B), RP (C), SPI (D), WPI (E) and PPI (N) (F) during a heating and cooling cycle, including a 90°C hold. Data is the averaged result of triplicate analysis, with standard deviation displayed in grey.

3.3.2.25. Ultrastructure

The ultrastructure of all protein ingredient powders was examined using scanning electron microscopy (SEM) at 1000x magnification and is shown in **Figure 3.5**. Each of the protein

ingredients differ in morphology. BRP can be seen to have irregular shaped particles in globular forms in a variety of sizes with evidence of surface interruptions. PPI (P) presents larger spherical particles with dimple surfaces. WPI displaces smoother and rounder globular particles in various sizes, some with slight dents. SPI displays a range of different shapes and



sizes of irregular particles. RP shows shrunken and wrinkled structures while PPI (N) displays irregular shapes and uneven surfaces.

Figure 3.5: Scanning electron microscope (SEM) imaging of BRP (A), PPI (P) (B), RP (C), SPI (D), WPI (E) and PPI (N) (F) at 1000x magnification.

3.3.3. In-vitro digestion model

3.3.3.26. True ileal digestibility

Cumulative true ileal digestibility for all protein ingredients is presented in **Figure 3.6**. True ileal digestibility, as analysed in using the dynamic TIM model, represents the bioavailability of nitrogen (N) over time and allow the calculation of a single 'digestibility' value. As can be seen in **Figure 3.6**, in the first 15 min of digestion, all samples, with the exception of PPI (N), have similar amounts of available N. This trend continues through to 30 min, with available N in PPI (N) lagging behind. Moving ahead to the 60-minute timepoint, BRP, PPI (P) and WPI begin to show increased N bioavailability. This trend becomes more pronounced throughout the remaining timepoints during digestion. From T120 onwards, BRP, PPI (P), WPI and PPI (N) show similar N bioavailability while digestion appears slower in RP and SPI. True ileal digestibility over the entire digestion period (0-300 min) is displayed in **Table 3.5**. PPI (P) displays the highest N digestibility (98.6%) followed closely by WPI (94.9 %), PPI (N) (93.6%)

and BRP (90.1%). Values are significantly lower for RP and SPI, 71.9% and 79.6% N bioavailability, respectively.

Table 3.5: The true ileal bio-accessible nitrogen digestibility and digestible indispensable amino acid score (DIAAS) calculated as described in Equation 8, with limiting AA(s) indicated.

		BRP	PPI (P)	RP	SPI	WPI	PPI (N)
Digestibility (% bio-accessible N)		90.1 ± 2.3	98.6 ± 2.2	71.9 ± 1.5	79.6 ± 2.4	94.9 ± 0.5	93.6 ± 0.9
Adult (> 3 yrs)	DIAAS (%)	67.3 ± 0.23	39.8 ± 3.28	37.5 ± 8.34	38.3 ± 7.45	89.7 ± 0.78	68.2 ± 0.05
	Limiting AA	Lysine	Sulphur AA	Lysine	Sulphur AA	Histidine	Sulphur AA

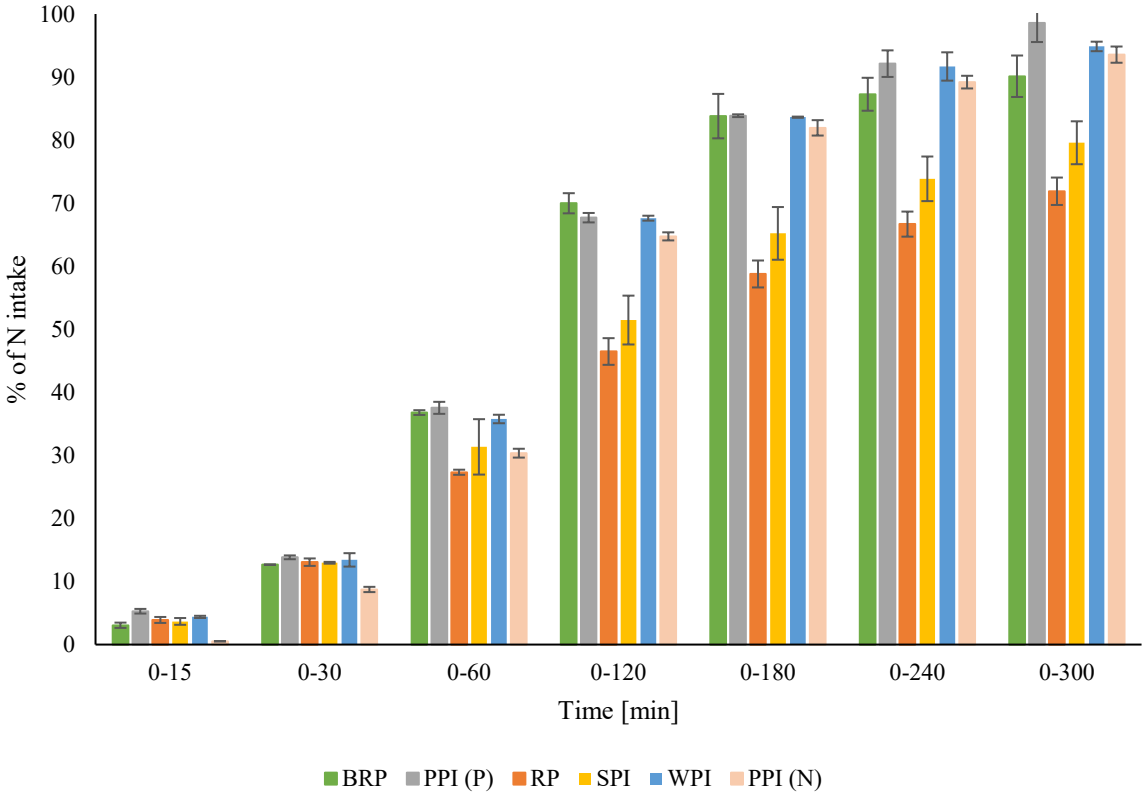


Figure 3.6: Cumulative true ileal digestibility of nitrogen (blank corrected) for all test products of time during 5 hours in tiny-TIMsg, expressed as the % of the exogenous nitrogen intake (corrected for nitrogen in gastric residue).

3.3.3.27. Protein Quality – Digestible indispensable Amino acid score (DIAAS)

The digestible indispensable amino acid scores (DIAAS) for each protein ingredient are displayed in **Table 3.5**, alongside the corresponding limiting amino acid. DIAAS values were calculated using true ileal bio-accessibility data for each individual essential amino acid and

reference values for an adult population. Limiting amino acids were the same for child and adult populations. Due to the expected application of BRP in food for those above the age of 3 and the applied digestion parameters, adult DIAAS are seen as the most relevant for discussion. It can be concluded that RP and SPI contained the lowest levels of bio-accessible essential amino acids, which corresponds to both of these ingredients having low overall N digestibility, and low adult DIAAS of 38% in both cases. PPI (P), although having the highest N digestibility, is limited by the sulphur containing amino acids, giving a low DIAAS of 40%. The remaining protein ingredients display higher values, with BRP and PPI (N) displaying DIAAS of 67% and 68% respectively, the highest of the plant-based protein sources examined in this study. WPI protein presents the highest DIAAS of 90%, with the limiting amino acid being histidine.

The digestible indispensable amino acid (DIAA) ratios of all samples are presented in **Figure 3.7.** A 'good-quality' source is indicated by exceeding the threshold line at 0.75, as recommended in the FAO report on dietary protein quality (2013). A DIAA of greater than 1 indicates a sufficient supply of the given amino acid. Regarding BRP, the DIAA exceeds 1 for all essential amino acids with the exception of the limiting amino acid lysine. This is similar for WPI and PPI (N), where the only essential amino acid not meeting the requirement of the reference pattern is the first limiting amino acid; histidine in the case of WPI, and the sulphur containing amino acids and tryptophan for PPI (N). However, in WPI histidine still falls above 0.75. At the other end of the spectrum, RP and SPI exhibit a DIAA below 1 for almost all essential amino acids, with many under 0.75. PPI (P) lies between these two extremes with only tryptophan falling short of the 0.75 benchmark, in addition to the limiting amino acids.

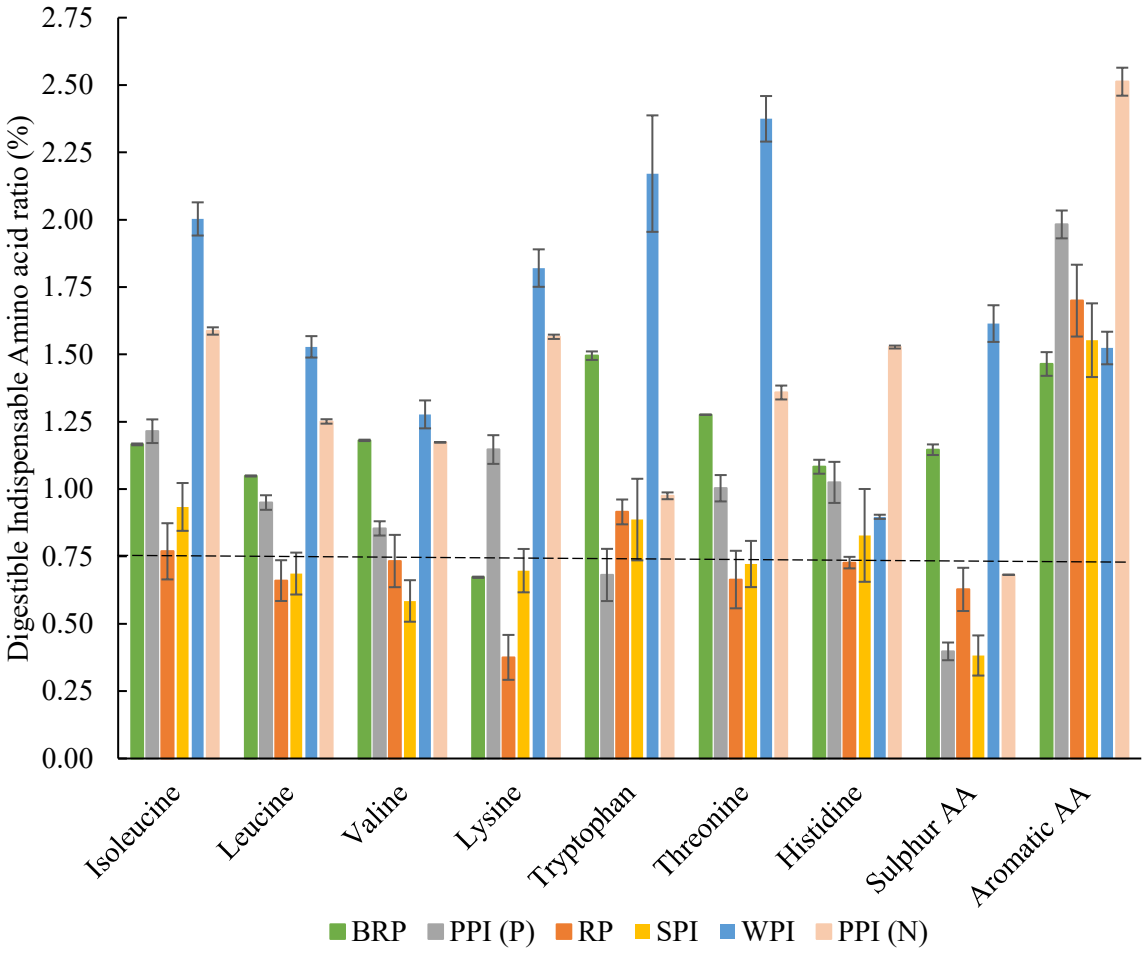


Figure 3.7: DIAA reference ratio over 5 hours of digestion in tiny-TIM, line at 0.75 and indicates a 'good' content of the essential amino acid while great while greater than 1 indicates a 'high-quality' protein. Calculated using >3 yrs reference values (FAO, 2013).

3.3.4. Principal Component Analysis

Principal component analysis was performed to identify correlations within the obtained data (**Figure 3.8**). PPI (P) and SPI are particularly clustered together. DIAAS is significantly positively correlated with protein solubility at all pH values (pH 3; *p*-value: 0.03, *r*-value: 0.91, pH 5; *p*-value: 0.01, *r*-value: 0.95, pH 7; *p*-value: 0.02, *r*-value: 0.94), as well as exposed (*p*-value: 0.00, *r*-value: 0.98) and free -SH-groups (*p*-value: 0.002, *r*-value: 0.98). Foaming capacity was found to be significantly positively correlated with separation rate (*p*-value: 0.03, *r*-value: 0.91), whereas it was positively correlated but not significantly to protein solubility at all pH values (pH 3; *p*-value: 0.11, *r*-value: 0.79, pH 5; *p*-value: 0.09, *r*-value: 0.81, pH 7; *p*-value: 0.17, *r*-value: 0.72).

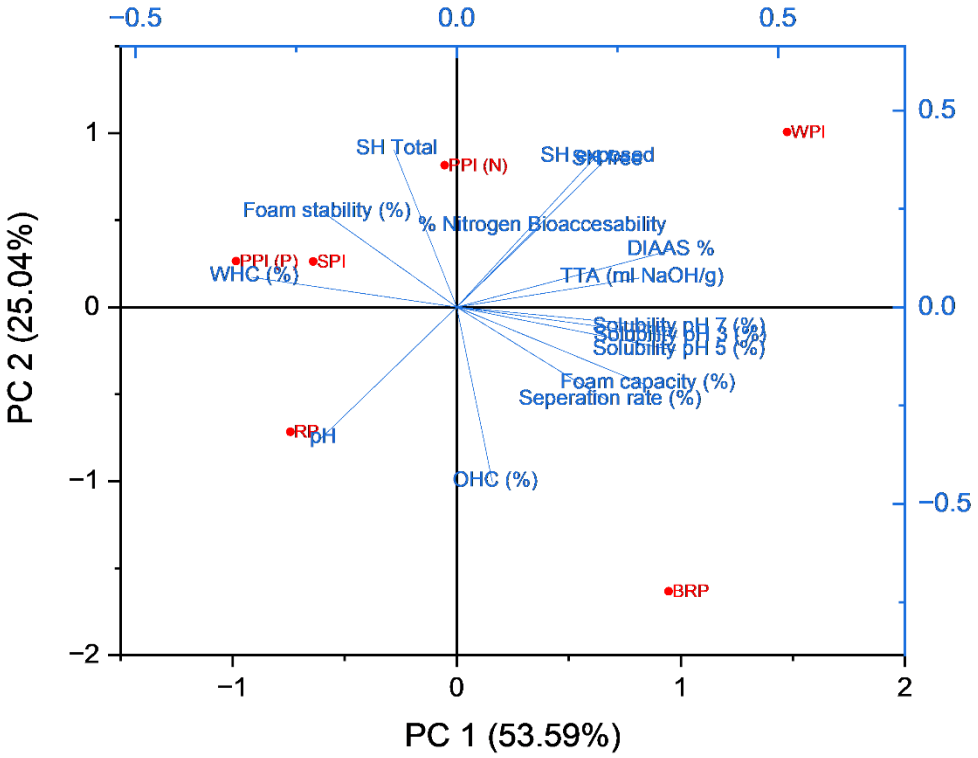


Figure 3.8: Principal Component Analysis biplot of the techno-functionality including pH, total titratable acidity (TTA), foaming, water and oil holding capacity (W/OHC), separation rate (%/min), sulfhydryl groups (SH) and protein solubility with digestion properties including (%) nitrogen bio-accessibility and digestible indispensable amino acid scores (DIAAS) of the protein ingredients. Barley and rice protein (BRP), Pea protein isolates PPI(P) and PPI (N), rice and soy protein isolates (RP), (SPI) and whey (WPI).

3.4. Discussion

This study examines the nutritional and functional properties of six protein isolates; five from well-known and extensively studied sources (whey, pea, rice, soy), and one from a novel source, brewers spent grain. As expected of isolates, all ingredients contained a high level of protein. The accurate measurement of protein in foods is of crucial importance regarding determining ingredient value, as well as for the formulation of foods, particularly those intended for nutritionally vulnerable sub-groups such as infants and the elderly. In an industrial setting, protein is usually determined indirectly by initial nitrogen quantification followed by protein calculation using a conversion factor. While acceptable and generally used as standard, these methods are prone to protein overestimation due to the inclusion of non-protein nitrogen as well as the use of the common conversion factor 6.25 for many proteins. Nitrogen content of proteins alters drastically based on protein source, and can even vary greatly within the same sources, depending on a wide variety of external factors. There also arises the issue of novel protein sources, such as BRP, where the suitable conversion factor is unknown, and research in this area is limited. In this study, protein content was determined by amino acid analysis as recommended by the FAO (2003) and these values, alongside N as determined by the Kjeldahl method, were then used to calculate the conversion factor K_p (Mossé, 1990). For all except WPI, the calculated conversion factors are lower than the standard conversion factor of 6.25. Interestingly, for BRP the conversion factor is higher than for the other plant proteins. In addition, the conversion factor for BRP exceed that of its individual components, which can be found in literature as 5.5 and 5.17-5.26 for barley and rice respectively (Fujihara et al., 2008; Mossé, 1990).

Fat levels are low in all samples, with the exception of PPI (P) and PPI (N). Higher levels of lipids can negatively affect the foaming behaviour of proteins (Lam et al., 2018), which can be observed in this study, with both pea proteins having the lowest foaming capacity. Differences in composition, including carbohydrates, ash, and sodium contents, can likely be attributed to differences in production processes, such as enzymatic treatments, filtrations, and precipitation steps. In particular, RP contains higher levels of starch and sucrose when compared to the other analysed ingredients. This may be due to reduced use of starch solubilising enzymes such as gluco-amylases and α-amylases during the extraction process (Jayaprakash et al., 2022), although the exact process is unknown.

Protein solubility is a key factor in protein digestion, as an increase in protein aggregation can inhibit proteolysis (Loveday, 2023). BRP displays a very high solubility (> 91 %) across the pH range tested, as was also observed in a previous study (Jaeger et al., 2023). This could be due to the enzymatic hydrolysis treatment (Nyhan et al., 2023), as is also indicated by abundance of low MW peptides in SDS PAGE electrophoresis. In fact, the only visible band for BRP is at ~ 39 kDa, potentially identifiable as Protein Z, a major barley albumin known to be resistant to enzymatic degradation (Jaeger et al., 2021). Enzyme hydrolysis may also be responsible for the increased solubility of PPI (N) over PPI (P). Protein solubility was also high for WPI over the pH range, although there was a decrease at pH 5. This slight decrease may be due to β-lactoglobulin or α-lactalbumin, the major proteins in whey and the most abundant bands in **Figure 3.3**, having an isoelectric point of ~ pH 5 (Goulding et al., 2019). A similar trend was observed for several of the other proteins, particularly SPI, where solubility reduced at pH 5, the isoelectric point lying at approximately pH 4- 5 (Karaca et al., 2011; Lam et al., 2018; Liu et al., 2010). PCA indicated that protein solubility is positively correlated with DIAAS, foaming capacity and separation rate.

Surface hydrophobicity can be linked to protein solubility, and therefore protein digestibility. A high degree of surface hydrophobicity can promote protein-protein interactions, increasing the tendency of a material to aggregate and potentially inhibit solubility (Carbonaro et al., 2012; Shevkani et al., 2019). Inversely, a lower surface hydrophobicity promotes protein-water interaction, promoting solubility. Although it was not possible to obtain a valid result for BRP regarding hydrophobicity, from the obtained data it can be hypothesised that the surface hydrophobicity is negligible, likely due to the lack of suitable 8-anilino-1-naphthalenesulfonic acid (ANS) binding sites (Cardamone & Puritt, 1992), potentially as a result of protein degradation as was discussed in a previous study (Jaeger et al., 2023). A hydrophilic nature would promote protein solubility, as can be observed with BRP and WPI. Increased solubility may, in turn, enhance proteolytic access and therefore lead to increased digestibility. RP and SPI both have a relatively high surface hydrophobicity, low protein solubility and exhibit the slowest N digestibility, the lowest overall N digestibility and a low DIAAS.

Protein solubility also significantly influences techno-functional properties, in particular foaming and emulsifying characteristics. These properties rely greatly on the ability of a protein to be active on the border between the protein and the associated water or oil. In some instances, an increased surface hydrophobicity and increased fat content, as observed for PPI (P) and PPI (N), can indicate improved surfactant properties. However, a review by Van Vliet et al. (2002)

outlines the relevance of processes involving pH change or protein conformational change on the interfacial behaviour of proteins and many descriptions of this effect in literature are contradictory (Lam et al., 2018). In the current study, it was observed through PCA that increased protein solubility was related to increased foaming capacity and a quicker emulsion separation rate. A higher content of fat present in the initial pea protein powders may also indicate an increased ability of the proteins to bind fat molecules. Therefore, for applications such as milk-style beverages which require emulsifying power the pea protein sources appear to be the most appropriate while BRP and WPI may require additional emulsifying agents. However, BRP and WPI, having a higher protein solubility than the other samples in this study, exhibit a minimal degree of sedimentation under accelerated stability analysis which is a desirable trait in beverage applications.

This study also examined the rheological properties of the chosen protein isolates over a heating and cooling cycle. This gives beneficial information regarding the implementation of such ingredients in food applications, where changes in structure during heating may be desired or are to be avoided. Gel strength can be influenced by protein concentration and the degree of protein denaturation, as well as the concentration of sulfhydryl groups in a protein matrix, where di-sulfhydryl crosslinking is one of the major factors responsible for stabilising gel matrices (Havea et al., 2009). Whey protein in particular is known for forming a strong, irreversible gel on heating due to the denaturation of whey proteins, mainly β-lactoglobulin, and the formation of di-sulphide linkages and other interactions. In this study, the final G' values of each protein correspond to the concentration of exposed and free sulfhydryl groups, with those with a higher concentration displaying a higher overall G' at the end of the heating and cooling cycle. The examined rheological properties can also be linked to the WHC, with PPI (P) and SPI exhibiting the highest WHC and also the highest initial G'. This thickening effect, in the absence of heating, may be useful in product formulations requiring thickening or cold-set gels. Alternatively, RP, WPI and PPI (N) show varying degrees of thickening on heating and may be suitable for use in sauces and soups, meat and cheese alternatives, and pudding type desserts. In addition, RP was the only ingredient to contain significant amounts of starch which may also influence the rheological properties. However, further studies into gel characteristics such as elasticity and brittleness would be beneficial.

The surface morphology of the protein, as observed using scanning electron microscopy imaging, displays a variety of particle shapes and sizes. PPI (P) and SPI appear to be similar, with a collection of dimpled globular individual bodies, whereas PPI (N) appears as a more

homogenous structure, potentially as a result of protein hydrolysis as seen in **Figure 3**. Interestingly, BRP has a different appearance than observed in a previous study (Jaeger et al., 2023), with the additional presence of small holes in the particles. This variation is likely due to batch or processing parameter variation. The presence of these small holes may be evidence of enzymatic hydrolysis treatment (Nyhan et al., 2023), as supported by the absence of protein bands in Fig. 3 and the high protein solubility .

Literature shows that plant proteins generally have a lower protein digestibility rate than animal-derived proteins, that is, because they tend to be more resistant to proteolysis (Bessada et al., 2019; Kaur et al., 2022; Mariotti, 2017). This can be observed in the current study, particularly with regards to RP and SPI. Protein digestion can be influenced by a variety of factors including integral protein structure and the presence of cell walls or membranes, processing parameters, and the presence of anti-nutritional compounds (Becker & Yu, 2013; Sá et al., 2020). The structure of the food matrix will greatly affect both digestion kinetics and overall digestibility. Particularly in plant-based sources, proteins may be shielded from digestive enzymes by the presence of a poorly permeable cell wall or membrane (Becker & Yu, 2013; Sá et al., 2020), although in protein isolates this effect is likely minimal due to increased ingredient purity as a result of the isolation process. What is more probable is that digestion is inhibited by protein structure or protein aggregation therefore limiting enzymatic access and influencing protein solubility. β-sheet structures are the major protein conformation type present in legume proteins and play a major role in decreasing protein digestibility due to an inhibition of protease activity (Carbonaro et al., 2012, 2015; Guo et al., 2019). In addition, pH changes during digestion may also affect protein aggregation and therefore digestion kinetics (Zhang & Vardhanabhuti, 2014). Grains and legumes are known to contain significant levels of anti-nutritional compounds such as phytic acid, trypsin inhibitors, tannins and lectins (Bajpai et al., 2005; Kaushal et al., 2012; Kumar et al., 2022) which may also be contributing to this lowered level of protein digestion. It may be of interest in future studies to examine BRP for these compounds.

Aside from RP and SPI, the other plant proteins in this study have a high N digestibility (>90%), regardless of their source. This may be due to certain processing parameters during isolation that alter protein structure or reduce the effect of antinutritional factors, through protein denaturation or degradation, respectively. Simple thermal processes such as cooking, autoclaving, or microwaving have been shown to increase protein digestibility and reduce the impact of anti-nutritional factors (Boye et al., 2012; Park et al., 2010; Vagadia et al., 2018).

Potentially, chemical processes such as glycosylation may also affect digestibility. Glycosylation is an early stage step in the Maillard reaction sequence causing the covalent conjugation of a free carbonyl group and the amine group of a protein, peptide or amino acid, and may induce structural changes, or unfolding, that has been described as increasing protein digestibility (Kaur et al., 2022) as well as altering functional and allergenic characteristics (Kutzli et al., 2021; Shao et al., 2020).

However, when considering protein quality in foods, N digestibility is only one of multiple factors to consider. DIAAS (Digestible Indispensable Amino Acid Score) is the successor to PDCAAS (Protein Digestibility Corrected Amino Acid Score) and is the preferred method for determining protein quality, with regards to the bioavailability of the essential amino acids (FAO, 2013). In a recent study by Herreman et al. (2020), the DIAAS of common plant and animal based proteins were compiled and compared using datasets collected from literature, specifically using pig models, giving a comprehensive overview of the average DIAAS ranges for various protein sources (Herreman et al., 2020). These largely agree with the findings observed in this study with similar values for rice, whey, and pea-based proteins and corresponding first limiting amino acids for all sources. In addition, in-vivo studies completed in a more recent pig (Bailey et al., 2023) and rat (Rutherfurd et al., 2015) models also show similar DIAAS values for rice, pea and whey. However, the DIAAS for SPI and PPI (P) are significantly lower than what is described in literature (Bailey et al., 2023; Van den Berg et al. 2022). In a review examining the DIAAS of a wide variety of soy products by Van den Berg et al. (2022), the mean adult DIAAS for soy is given as 84.5 ± 11.4, and Herreman et al. (2020) propose an average DIAAS > 100 for soy protein. This significant gap could be due to treatments applied to this specific soy protein ingredient during processing (eg alkaline or heat treatment) leading to protein aggregation or structural change as described previously, or might be a result of differences in the innate protein structure due to cultivar or growing conditions (Gilani & Sepehr, 2003; Kaur et al., 2022; Vasconcelos et al., 2010). This is further backed by the overall low (<50 %) bioavailability of the majority of amino acids in SPI, and a low N digestibility. The same can be said of PPI (P). In addition, differences in methods of determination are likely also be contributing to this effect. In this particular study, it can also be noted that the isolates with a low DIAAS also exhibited a lower protein solubility and increased protein aggregation, potentially inhibiting digestion. Interestingly, this in not true for PPI (P) which shows a high N digestibility. PCA also shows this, with DIAAS being positively correlated to protein solubility as well as N bio-accessibility. Although minimal studies exist

on the digestion characteristics of BRP, it is interesting to note that the DIAAS obtained for BRP exceed those of its components, barley and rice, that can be observed in literature where the DIAAS values range from 40-56 % (Cervantes-Pahm et al., 2014; Fu et al., 2023; Nosworthy & House, 2017) and 37-52 % (Bailey et al., 2023; Hertzler et al., 2020; Rutherfurd et al., 2015), respectively. While Lysine is the limiting amino acid for both protein sources, this increase is likely due to BRP being a refined and hydrolysed food ingredient.

A recently published study examines the amino acid uptake behaviour of BRP in 12 participants (Ummels et al., 2023). The estimated total amino acid uptake for BRP was 69 % and 87 % as a percentage of whey and pea protein PPI (N), respectively. This trend is similar to those observed in the present study, specifically regarding N % digestibility and DIAAS values, with WPI displaying the highest digestibility and DIAAS, followed by PPI (N) and BRP respectively.

3.5. Conclusion

This study outlines the digestibility and functional behaviour of five widely used protein isolates and introduces a novel protein isolate extracted from brewers spent grain. This new protein displayed a high protein solubility, similar to WPI, as well as a high N digestibility (> 90 %), and foam capacity. However, over heating BRP alone displayed no rheological changes. BRP may be useful in beverage applications where pasteurisation or similar treatments are required, without changes in product stability. As previously seen in literature, this study shows that many of the plant-based proteins were nutritionally inferior to whey, with PPI (P), RP and SPI displaying low DIAAS vales (< 40 %). However, regarding BRP, the DIAA for all essential amino acids was >1 with the exception of lysine giving a DIAAS of 67 %. In combination with a N digestibility >90 %, the data shows that BSG protein is a nutritious protein. The combination of BRP with other protein sources, e.g. pea protein isolate as examined in this study, may be the solution, altering both the nutritional and functional characteristics depending on the specific requirements of the food product. The implementation of such proteins in different food matrices likely affects protein digestibility and so further study in this area is required. However, the commercial availability of such a protein ingredient gives food manufacturers the ability to begin the shift towards a circular food system and is an important step in the journey towards an increasingly sustainable food future.

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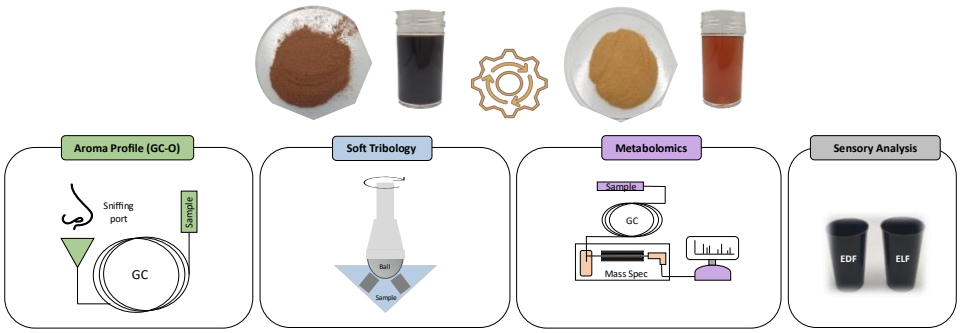
4. Chapter 4

Decolourised barley and rice protein isolate: Enhanced techno-functional and sensory properties



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Abstract

Plant-based protein is increasingly preferred over animal-based protein from an environmental and ethical standpoint. EverPro®, a barley and rice protein isolate (BRPI), upcycled from brewers spent grain is a commercially available novel plant protein. The aim of this study was to compare two BRPIs, EverPro® Dark Fraction (EDF) and EverPro® Light Fraction (ELF), which underwent an additional decolourisation process. Both ingredients showed high solubility values over a range of pH values which were found to be linked with their zeta potential. Other techno-functional properties were also analysed, where only slight differences between ingredients were found. Key differences between EDF and ELF were colour and sensory characteristics. ELF showed a significantly higher L* values (beige/sandy) compared to EDF (dark brown). Moreover, a major decrease in aroma and metabolite compounds was observed for ELF. The sensory analysis revealed ELF was perceived significantly lower in bitter, burnt/roasty descriptors in comparison to EDF. These findings correlated with a change in tribology measurements, specifically, an increase in lubrication of ELF solution compared to EDF. The increase in lubrication resulted in a higher viscosity perception by the panellists. Overall, these findings highlight the extended potential of EverPro® with enhanced physical properties for application in a wider range of food and beverage systems.

4.1. Introduction

With the global population projected to reach 9.5 billion by 2050, this is forecasted to lead to a greater increase in the demand for dietary proteins (United Nations, 2015). Additionally, trends are moving away from animal-based diets as individuals seek to incorporate more plant-based foods for ethical, environmental, and health-related reasons (Henchion et al., 2017). Because of this, interest in new and emerging sources of plant-based proteins is on the rise. Some common plant-based protein ingredients currently popular on the market include soy, pea, wheat, and rice, with their popularity likely due to the extensive research and knowledge associated with these ingredients (Hoehnel et al., 2022). However, from a sustainability point of view, the valorisation of protein ingredients from food processing side streams holds significant importance. This approach aims to reduce food waste, enhance resource efficiency, and contribute to a circular economy. A study by the Good Food Institute showed that the top side-stream candidates for the production of protein hydrolysates included soy, corn distillers dried grain with solubles, canola and corn gluten meal and barley brewers spent grain (Eastham et al., 2023). These side streams show great potential for producing alternative proteins to address future protein demands.

Barley is the most commonly used raw material for the brewing process, often used in combination with adjunct grains such as rice. Adjuncts are used to replace a portion of barley malt as alternative sources of fermentable extract in the wort. Additionally, adjuncts can enhance beer characteristics and quality such as colour, flavour and foam. Rice, when used as an adjunct gives a light flavour to larger beers and is the second most widely used adjunct in the United States (Stewart, 2006). Brewers spent grain (BSG) is the residual material from the initial brewing grains following the malting and mashing processes of beer making. It is the most abundant by-product of the brewing industry, constituting 85% of total brewery coproduct with approx. 20 kg of wet BSG produced from 100 litres of beer (Jaeger et al., 2021; Mussatto et al., 2006). BSG is not only abundant in fibre but also has a high protein content, with the protein fraction ranging from 19 – 30%, where 30% of this total protein consists of essential amino acids, with lysine being the exception. The primary proteins found in barley include hordeins and glutelin. The main protein in rice is a glutelin type storage protein (Jaeger et al., 2021). The rich dietary fibre and protein content of BSG can be considered a promising side stream for valorisation into a functional food ingredient (Eastham et al., 2023). Additionally, upcycling of BSG is of interest to the brewing industry as it can be repurposed into more

sustainable food systems that minimises waste and maximises residue utilisation. BSG has already been successfully applied in a variety of food systems to enhance their nutritional value, including bread, pasta, muffins, yoghurt, and frankfurters (Naibaho et al., 2022; Nocente et al., 2019; Özvural et al., 2009; Sahin et al., 2021). However, while the incorporation of BSG can significantly elevate the nutritional profile of a food, this is only possible up to a level, after which the negative quality characteristics of the food ingredient offsets any nutritional benefits (Nyhan et al., 2023).

The colour of a protein ingredient can be an important attribute for several reasons such as visual appeal and consumer expectations. Ingredients with a colour closer to white can be incorporated more easily in a wider range of products and increase its flavour profile. Consumers anticipate specific tastes and flavours based on colour of food and beverages. When the actual taste or flavour doesn't align with the expected colour, it can lead to consumers perceiving the product as unacceptable (Spence, 2015). Differences in colour properties can arise from the origin of the plant protein due to the presence of coloured pigments. The removal of colour from protein ingredients, or the decolourisation, can be achieved through various means of chemical and enzymatic bleaching, ultraviolent radiation alongside different processing techniques. Therefore, the decolourisation of these ingredients might improve their application to certain food and beverages, where colour is a concern. However, these methods of decolourisation need to be carefully considered, as they can also impact functional and flavour characteristics (Carter & Drake, 2018; Shen et al., 2021).

Chemical, biological and physical processes can be used to extract the protein fraction of a raw material, while also modifying functional properties and potentially improving the technological performance of an ingredient in different food systems (Munialo, 2024). For example, proteins extracted using enzymatic methods have been shown to exhibit thermal stability, low viscosity, and improved functional properties such as foaming, solubility and emulsion stability (Kumar et al., 2021). Furthermore, specific protein modification methods can effectively diminish or eliminate unwanted antinutrients, while also masking undesirable organoleptic properties (Munialo, 2024). A limited number of studies have been carried out on the functional characterisation and application of protein isolates extracted from BSG (Celus et al., 2007; Connolly et al., 2014; Jaeger et al., 2023), with results showing enhanced techno-functional properties including improved solubility, foam capacity and stability along with better emulsion forming ability.

EverPro® Dark Fraction (EDF), manufactured by the company EverGrain, is a barley and rice protein isolate (BRPI), extracted from the brewers spent grain obtained from a brew using malted barley and rice adjunct and therefore contains a mixture proteins from both cereals. These ingredients are commercially available largely in the United States (US) and Europe (EU). The ingredient is produced by the concentration of proteins from barley and rice BSG using a series of enzymatic hydrolysis and sized-based separation and purification steps (Turck et al., 2023). Studies by Jaeger et al. (2023, 2024) compared the techno-functional properties and digestibility of EDF to other plant-based protein isolates such as pea, soy and rice, along with an animal-based protein (whey), with results highlighting the similarity of EDF to whey with regards to solubility, nitrogen bio-accessibility and nitrogen digestion kinetics. These studies also concluded EDF exhibited extensive protein hydrolysis, as indicated by its protein profile, surface hydrophobicity and viscoelastic behaviour, suggesting the ingredients' structure is primarily composed of small peptides and free amino acids (Jaeger et al., 2024). EverGrain recently expanded its ingredient portfolio with the launch of an EverPro® Light Fraction (ELF), a new BRPI produced by further processing of EDF. The impact of this additional processing on the physicochemical and nutritional properties of ELF has not yet been characterised. Therefore, this study aims to compare the appearance and powder characteristics, composition, protein biochemistry and techno-functional properties of EDF and ELF, providing insight to potential applications in food and beverages systems.

4.2. Materials and methods

4.2.1. Materials

Barley and rice protein isolates, EverPro® Dark Fraction (EDF) and EverPro® Light Fraction (ELF) were obtained from EverGrain Ingredients (St. Louis, MO, USA). Chemicals for the analyses were supplied by Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise.

4.2.2. Compositional analysis

McCleary & McLoughlin, (2023),McCleary & McLoughlin, (2023),Compositional analysis was carried out externally by Chelab S.r.l. (Resana, Italy) including: Moisture, protein, fat, ash, minerals and the calorie content, based on the AOAC and ISO standard methods. Fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) were determined using high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) performed on a DionexTM ICS-5000+ system (Sunnyvale, CA, USA), as described by Ispiryan et al. (2019). Sugars were determined by high performance liquid chromatography, using the extraction method of Hoehnel et al. (2020). Total starch was determined in duplicate using the Megazyme kit K-RAPRS (Bray, Ireland). Total and insoluble dietary fibre, as well as soluble high and low molecular weight fibre was measured using AOAC 2022.01 according to McCleary & McLoughlin, (2023), with slight modifications where samples were deionised three times instead of two. All fibre was measured in triplicate except for soluble low molecular weight fibre (SDFS) which was measured in duplicate.

4.2.3. Powder characteristics

4.2.3.1. Colour and ultrastructure

The CIE (XYZ) of EDF and ELF powders was measured to using the Hunter colour system CIE L* a* b* and RGB colour values as described by Neylon et al. (2023)**.** This was measured using a handheld Konica Minolta colorimeter (Chroma meter CR – 400, Konica Minolta, Osaka, Japan) set at illuminant D65 where three measurements were taken in triplicate. The colour index for L* represents black to white (0 to 100), a* from green to red (-120 to 120) and b* from blue to yellow (-120 to 120). The instrument was calibrated beforehand using a calibration tile.

Scanning electron microscopy (SEM) was used to examine the ultrastructure of the ingredients according to the method reported by Atzler et al. (2021). The following settings were applied for the analysis: 5 kV voltage, 20 mm working distance and a magnification factor of both 50x and 1000x.

4.2.3.2. pH and total titratable acidity (TTA)

Ten grams of ingredient was mixed with 95 ml of deionised water and 5 ml of acetone and stirred using a magnetic stirrer. The pH was determined at room temperature using a pH meter (Mettler Toledo, Ohio, United States). Total titratable acidity (TTA) was measured as described by Jaeger et al. (2023) by titration of the solution with 0.1 M NaOH until a pH of 8.5 was reached. TTA is presented as ml of 0.1 M NaOH/g of sample.

4.2.3.3. Particle size

Particle size distribution was measured over a size range of 0.01 – 3000 μm, using a static light diffraction unit (Mastersizer 3000, Malvern Instruments Ltd, Worcestershire, UK). Samples were measured on a dry basis. An absorption of 0.001 and a particle refractive index of 1.45 were used for both samples. Results shown in D[4,3], refers to volume weighted mean particle size (i.e. reflects the size of the particles which make up the bulk of the sample) and D[3,2] refers to surface weighed mean particle size (i.e. relevant where specific surface area is important). D[10] shows less than 10%, D[50] less than 50%, D[90] less than 90% of the sample's average particle size.

4.2.3.4. Metabolomics

Metabolomic analysis was carried out by MS-Omics (Vedbaek, Denmark). Briefly, samples were derivatised with methyl chloroformate using a slightly modified version of the protocol described by Smart et al. (2010). All samples were analysed in a randomized order. Analysis was performed using GC (gas chromatography) (7890B, Agilent, CA, USA) coupled with a quadrupole detector (5977B, Agilent, CA, USA). The system was controlled by ChemStation (Agilent, CA, USA). Raw data was converted to netCDF format using Chemstation (Agilent, CA, USA) before the data was imported and processed in Matlab R2021b (Mathworks, Inc., Natick, MA, USA) using the PARADISe software described by Johnsen et al. (2017). Samples were analysed for 31 typical metabolites from the TCA (tricarboxylic) cycle.

4.2.4. Protein biochemistry

4.2.4.5. Amino acid composition

Amino acid analysis was carried out externally by Chelab S.r.l. (Resana, Italy) using ion chromatography with post-column derivatisation with ninhydrin or HPLC-UV analysis in the case of tryptophan.

4.2.4.6. Protein profile analysis

Protein profile analysis was performed using Agilent Bioanalyzer 2100 lab-on-a-chip capillary electrophoresis system and an Agilent Protein 80 kit due to high level of small peptides in the samples (AgilentTechnologies, St. Louis, USA). Additionally, the molecular weights of the respective protein bands were determined by comparing them to a molecular weight ladder. Samples were prepared as described by Vogelsang-O'Dwyer et al. (2020) with minor modifications. Briefly, EverPro ingredients were dispersed in 2% dithiothreitol (DTT), 2 M thiourea and 6 M urea (Bayram & Alameen, 2018) to obtain a protein concentration of approximately 1.5 to 1.8 mg/mL. Dispersions were shaken for 2 h at 22°C and analysed according to the Agilent Protein 80 kit instructions within the ranges of 5–80 kDa under reducing conditions.

4.2.4.7. Protein solubility

Protein solubility at various pH values was determined according to Alonso-Miravalles et al. (2019) with modifications. Sample dispersions of 1% (w/w) protein were prepared in deionised water followed by pH adjustment to pH 2, 4, 7 and 9 using 0.1 – 1 M HCl or NaOH. The dispersions were shaken at 500 rpm overnight at 4°C and then centrifuged at 4863 rcf for 30 min at 20°C. The protein content in the supernatant was measured using the Kjeldahl method AACC 46-12 (AACC International, 2011) and calculated based on a nitrogen to protein conversion factor of 6.25 for both ingredients. Protein solubility was expressed as the % of protein remaining in the supernatant.

4.2.4.8. Zeta potential

Zeta potential of the ingredients was determined using the Zetasizer Nano-Z (Malvern Instruments Ltd., Worcestershire, UK) as described by Vogelsang-O'Dwyer et al. (2022). Samples of 0.1% (w/v) protein dispersions were prepared using deionised water and adjusted to pH 2, 4, 6 and 8 using 0.1 – 1 M HCl or NaOH. Samples were hydrated by shaking at 500 rpm overnight at 4°C. Measurements were performed at room temperature using automatic

voltage selection, a refractive index of 1.45 and an absorbance of 0.001. Zeta potential was calculated using the Smoluchowski model.

4.2.4.9. Sulfhydryl groups

Exposed, free and total sulfhydryl (SH)-groups were measured using Ellman's reagent [5,5′-dithio-bis-(2-nitrobenzoic acid)] as described by Alonso-Miravalles et al. (2019) with some adjustments. For determination of exposed SH-groups, 75 mg of sample was extracted overnight at 4°C in 10 ml Tris- Glycine buffer at pH 8 consisting of 0.1 M Tris, 0.1 M glycine and 4 mM EDTA. 80 µl of Ellman's reagent was added to 2.5 ml of the protein dispersion and a reagent blank, where the protein sample was replaced by the Tris-Glycine buffer. Samples were incubated at room temperature for 15 min, centrifuged at 4863 rcf for 15 min at 22°C and absorbance at 412 nm was measured using a spectrophotometer. The same protocol was followed for the determination of total SH-groups, but 8 M urea was added to the Tris-glycine buffer, and no centrifugation was performed prior to the absorbance reading. For the measurement of total SH-groups, 50 µl of 2-mercaptoethanol (BME) and 4ml of Tris- Glycine buffer at pH 8 consisting of 0.1 M Tris, 0.1 M glycine, 4 mM EDTA and 8 M urea was added to 1 ml of protein dispersion and the solution was shaken at 500 rpm at room temperature for one hour. The supernatant was discarded to remove the BME, and the pellet resuspended twice in 12% TCA followed by centrifugation at 500 rpm for 15 min at 22°C. The pellet was resuspended in 10 mL of the Tris-Glycine buffer containing 8 M urea. 40 µl of Ellman's reagent was added to 4 ml of the diluted protein sample and a reagent blank and the solution were incubated at room temperature for 15 min, after which absorbance at 412 nm was measured. The content of SH-groups was calculated as follows:

$$\mu m \frac{SH}{g} protein = \frac{(\,(\,\mathrm{A\,412} - \mathrm{A\,412\,a} - \mathrm{A\,412\,b}) * 1000000 / \varepsilon)}{C}$$

Where A 412 is the absorbance at 412 nm, A 412a and A 412b are the absorbance values of the sample blank and the reagent blank respectively, ε is the extinction coefficient, which was taken as 13,600 M^{-1} cm^{-1}, and C is the protein concentration (mg/ml) of the diluted sample.

4.2.5. Techno-functional properties

4.2.5.10. Emulsion characteristics

Emulsion stability was measured as described by Jaeger et al. (2023). Emulsions of 1% (w/v) protein were prepared with deionised water and adjusted to pH 7 shaken overnight at 500 rpm.

10% sunflower oil was then added, after which the sample was sheared using an Ultraturrax equipped with a S10N-10G dispersing element (Ika-Labortechnik, Janke and Kunkel GmbH, Staufen, Germany), at speed 5 for 2 min. Emulsion stability was measured using an analytical centrifuge (Lumisizer, GmbH, Berlin, Germany) with parameters of 100 rcf for 15 min at 15°C, at a wavelength of 865 nm. Lumisizer software measures the percentage of light transmission over time. Emulsion stability is then expressed as the separation rate, which is the percentage of integral light transmission per minute.

4.2.5.11. Oil holding capacity

Oil holding capacity (OHC) was determined using the method described by Boye et al. (2010) with slight modifications. Briefly, 1 g of ingredient was mixed with 6 g of sunflower oil. The sample mixtures were allowed stand for 1 h for sufficient uptake of oil, followed by centrifugation at 4000 rcf at 20°C for 30 min. The supernatant was then carefully removed before the tubes were inverted for 30 min to allow sufficient drainage. OHC (%) was calculated using the following formula:

$$OHC\ (\%) = \frac{(Weight\ of\ tube + pellet) - (Weight\ of\ empty\ tube)}{Weight\ of\ ingredient} \times \frac{100}{1}$$

4.2.5.12. Foaming capacity and foam stability

Solutions of 2% (w/v) concentration were prepared with distilled water. The pH was adjusted to 7 using various concentrations of 0.1 – 1 M HCl and NaOH and the samples were hydrated overnight at 4°C. Briefly, samples were then frothed using an Ultra-Turrax equipped with a S10N-10G dispersing element (Ika-Labortechnik, Janke and Kunkel GmbH, Staufen, Germany) at maximum speed 6 for 30 s. The height of the sample (foam phase only) was measured immediately and after 60 min. Foaming capacity was measured as % sample expansion at 0 min, while foam stability was measured as the sample expansion at 60 min as a percentage of sample expansion at 0 min. Sample expansion was calculated using the equation as follows:

$$Foaming\ capacity\ (\%) = \left(\frac{Foam\ height\ immediately\ after\ foaming}{Initial\ sample\ height}\right) \times 100$$

$$Foam\ stability\ (\%) = \left(\frac{Foam\ height\ after\ 1\ hour}{Foam\ height\ immediately\ after\ foaming}\right) \times 100$$

4.2.6. Sensory attributes and tribology

4.2.6.13. Aroma profile of the powders

The aroma profiles of the ingredients were analysed externally by AromaLab (Aro-maLAB GmbH, Planegg, Germany) using gas chromatography- olfactometry (GC-O) with an intensity scale. The GC-O analyses were each performed once by two trained panellists. The panellist can rank the intensity on a scale of 0 to 3 in 0.5 steps, in which 0 = not detected, 1 = weakly detected, 2 = unequivocally detectable and 3 = intensity detectable. The intensity values are the mean value of the two panellists. Samples of 50 g were extracted with 100 ml of distilled diethyl ether and 100 ml of tap water. The organic layer was separated from the residue and the volatile compounds were isolated via safe distillation (solvent assisted flavour evaporation). The solution was dried over sodium sulphate and concentrated to 100 µl using a Vigreux column. Additionally, a trained sensory panel (n = 7) evaluated the aroma profile of each ingredient ortho-nasally and ranked them on an intensity scale from 0 (indiscernible) to 3 (strong discernible).

4.2.6.14. Descriptive sensory analysis of EverPro solutions

Sensory analysis of EDF and ELF was conducted by a panel of experienced sensory testers (n= 8, age range 23 - 35) recruited from the School of Food and Nutritional Sciences at University College Cork. Panellists were specifically trained for one day on the mouthfeel attribute, viscosity. This training involved using three different concentrations of EDF at 2.5%, 7% and 13% (w/w) representing low, medium, and high viscosity levels, respectively. Black sensory cups were used to prevent the perception of both dark and light colours from influencing the perceived viscosity of the beverages. During training each panellist was tested with unknown EDF beverage concentrations and asked to rank them based on viscosity. Following, aqueous testing solutions were prepared at a concentration of 10% (w/w). The panellists evaluated the intensity of the following descriptors on a scale of 0–10, with 10 indicating highly detectable (extreme) and 0 indicating undetectable (none): taste: bitter, salty, umami; flavour: burnt/roasted, caramel, soapy, earthy, malty/cereal; mouthfeel: viscosity. The definitions of the descriptors are displayed in **Supplementary Table 4.2**. Moreover, a rank order test on mouthfeel: viscosity, was conducted in which the panellist was given one sample each of EDF and ELF at 10% (w/w) and asked to determine the sample with the highest viscosity. A reference list defining each of the characteristics of interest was given to the panellists. The

sensory analysis took place in a sensory analysis room and was completed in triplicate across three separate sessions.

4.2.6.15. Tribology

Tribological measurements were conducted on an MCR301 using the BC-12.7 ball-on-3-pins tribology attachment (Anton Paar GmbH, Graz, Austria) at 37°C as described by Fox et al. (2022). The attachment contains changeable tribopairs (a glass ball and polydimethylsiloxane (PDMS) pins), which were supplied by Anton paar (Graz, Austria). New pins were used for each test consisting of one run-in period and two repeated measurements of Stribeck curves. Before starting a test, the pin-holder and ball-holder were washed gently using a dilute detergent solution and rinsed thoroughly as well as being rinsed in acetone and dried with lab-wipes with the tribopairs. The test sequence was as followed: first the glass ball was lowered until a target force of 3 N was reached and held at that height for an equilibration period of 1 min. Furthermore, the following settings of the measurement system were selected: a logarithmic increase in sliding speed from 10^{-8} to 1 m/s was performed recording 80 data points with point duration from 1 to 10 seconds logarithmic, followed by a resting period of 1 min. 10% (w/w) solutions of EDF and ELF were prepared using deionised water and measurements were conducted using 1 ml aliquots. Two consecutive measurements of Stribeck curves were performed. Three tests per sample were performed to ensure triplicates, the first measurement being the run-in period not included in the data set, resulting in six datasets per sample. Data was interpreted by the comparison of friction factors at set sliding speeds as previously described by Laiho et al. (2017) and Steinbach et al. (2014).

4.2.7. Statistics

Statistical analysis was only performed on data completed in triplicate or more. All statistical analysis was performed using IBM SPSS version 26 (Armonk, NY, USA) using an independent sample t-test with a statistical significance level of 5%. When data was not normally distributed a non-parametric independent Mann-Whitney U test was applied. Rank test statistical differences were done using a binomial test.

4.3. Results

4.3.1. Composition

The composition of EDF and ELF are presented in **Table 4.1**. The moisture value for EDF is slightly higher than ELF at 5.7 ± 0.21 g/100g and 3.9 ± 0.14 g/100g, respectively. As expected,

both isolates have high protein contents, with ELF slightly higher at 86.9 g/100g compared to EDF at 83.6 g/100g. Regarding carbohydrate content, EDF contains more than twice as much starch as ELF, but a lower content of total dietary fibre. Both ingredients contained low levels of sugars (<0.5 g/100g) and FODMAPs (<0.04 g/100g). Moreover, both EverPro ingredients are low in fat and have similar calorie contents of 351 ± 2 and 357 ± 2 kcal/100g for EDF and ELF, respectively. Regarding the mineral profile of the ingredients, ELF shows higher levels of calcium, iron, magnesium, manganese, and copper than EDF.

Table 4.1: Compositional analysis of EDF and ELF. Values in the same row that share a letter do not differ significantly ($p < 0.05$). DM – dry matter.

	EDF	**ELF**
Macronutrients		***g/100g***
Moisture	5.70 ± 0.21	3.89 ± 0.14
Protein (as is)	83.6 ± 3.20	86.9 ± 3.30
Fat (as is)	0.15 ± 0.02	0.06 ± 0.01
Carbohydrate (as is)		
Sugars (glucose, fructose, sucrose, maltose, lactose)	0.05 ± 0.01	0.03 ± 0.01
Total starch	2.68 ± 0.02	1.03 ± 0.04
Total Dietary Fibre	5.30 ± 0.43 [a]	5.76 ± 0.37 [a]
Insoluble dietary fibre (IDF)	0.83 ± 0.18 [a]	1.74 ± 0.22 [b]
Soluble high molecular weight fibre (SDFP)	2.99 ± 0.63 [a]	3.35 ± 0.43 [a]
Soluble low molecular weight fibre (SDFS)	1.24 ± 0.11	0.98 ± 0.03
Ash	6.69 ± 0.45	4.95 ± 0.33
Micronutrients	***mg/kg***	
Chlorides (as NaCl)	1500 ± 120	330 ± 25
Calcium	322 ± 48	1840 ± 260
Iron	9.8 ± 2.0	31.4 ± 6.2
Phosphorus	940 ± 180	860 ± 160
Magnesium	650 ± 130	2170 ± 410
Manganese	1.23 ± 0.28	1.60 ± 0.36
Potassium	10200 ± 1900	6700 ± 1300
Copper	6.8 ± 1.5	14.4 ± 3.0
Zinc	6.7 ± 1.3	5.0 ± 1.0
Sodium	13100 ± 2400	11800 ± 2200
FODMAPs (Total g/100g) (as is)	0.04 ± 0.01 [a]	0.03 ± 0.01 [a]
Energy (kcal)	351 ± 2	357 ± 2

4.3.2. Powder characteristics

4.3.2.16. Colour and ultrastructure

The colour values of the ingredients and solutions are presented in **Table 4.2**, as well as images of both ingredients and 10% (w/w) solutions of EDF and ELF are shown in **Figure 4.1**. ELF

powder showed significantly higher L* values at 72.70 ± 0.22 compared to EDF 53.15 ± 0.44 ($p < 0.05$), indicating a lighter coloured ingredient. The a* values showed that compared to EDF, the redness of ELF was significantly decreased, while the b* values showed that yellowness was significantly increased. Considering the RGB images, EDF has a darker colour (brown) compared to ELF (beige/sandy). **Figure 4.1** also shows the ultrastructure of EDF and ELF. The EverPro ingredients differed slightly in morphology. While both appeared to have irregular shaped particles in globular form with surface interruptions, EDF showed a large hole in the centre of the particle, evidence of damage (as seen in the magnification 50x shown in **Supplementary Figure 4.1** where EDF showed more evidence of this damage than ELF). Moreover, EDF appeared to have a rougher surface, while ELF showed a smooth covering film.

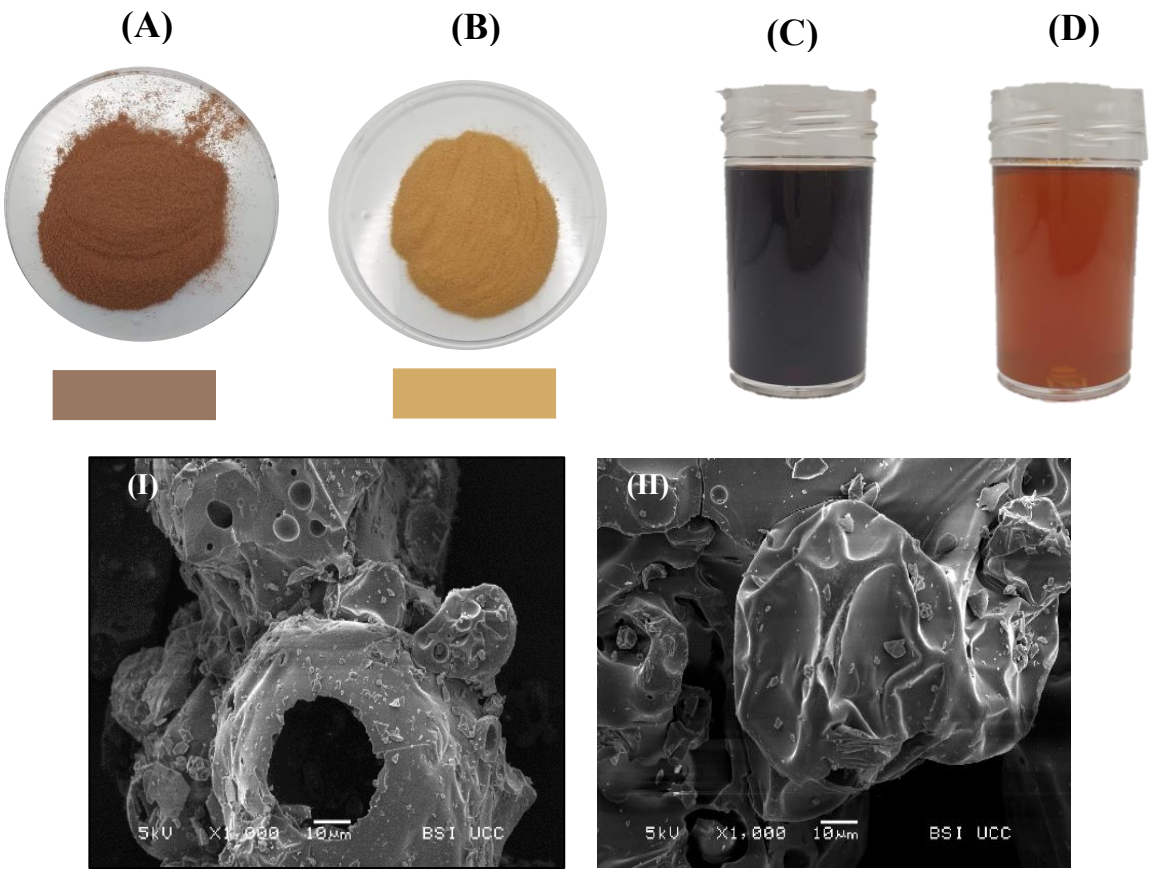

Figure 4.1: Images and RGB pictures of EDF (A) and ELF (B) in powder and EDF (C) and ELF (D) in a 10% (w/w) water solution. Scanning electron microscope (SEM) imaging of EDF (I) and ELF (II) at 1000× magnification.

Table 4.2: Powder characteristics and techno-functionality of EDF and ELF. Values in the same row that share a letter do not differ significantly ($p < 0.05$).

	EDF	**ELF**
Powder characteristics		
Colour L*	53.15 ± 0.44 [a]	72.70 ± 0.22 [b]
Colour a*	10.57 ± 0.20 [a]	7.79 ± 0.11 [b]
Colour b*	16.75 ± 0.12 [a]	40.82 ± 0.17 [b]
pH	7.80 ± 0.06 [a]	7.59 ± 0.03 [b]
TTA (ml 0.1 M NaOH/g)	1.10 ± 0.07 [a]	0.58 ± 0.02 [b]
Particle size D[3,2] (µm)	65.29 ± 7.13 [a]	102.98 ± 2.58 [b]
Particle size D[4,3] (µm)	180.56 ± 21.27 [a]	177.22 ± 4.24 [a]
Particle size Dv(10) (µm)	45.18 ± 9.41 [a]	67.51 ± 4.25 [b]
Particle size Dv(50) (µm)	149.44 ± 6.67 [a]	171.00 ± 3.00 [b]
Particle size Dv(90) (µm)	292.22 ± 6.67 [a]	296.56 ± 7.67 [a]
Techno-functionality		
Emulsion separation rate (%/min)	4.48 ± 0.28 [a]	4.78 ± 0.15 [a]
Oil holding capacity (%)	156.30 ± 10.52 [a]	185.42 ± 7.79 [b]
Foam capacity (%)	113.68 ± 6.20 [a]	114.63 ± 3.84 [a]
Foam stability (%)	35.31 ± 0.84 [a]	15.17 ± 1.31 [b]

4.3.2.17. pH and TTA

The pH and TTA values are presented in **Table 4.2**. Both pH values were similar, with EDF exhibiting a slightly but significantly higher pH (7.80 ± 0.06) than ELF (7.59 ± 0.03). EDF showed a significantly higher TTA value than ELF, with values of 1.10 ± 0.07 and 0.58 ± 0.02 mL of 0.1 M NaOH/g determined, respectively.

4.3.2.18. Particle size

The particle size results are shown in **Table 4.2**. Results showed that EDF has a much smaller D[3,2] particle size of 65.29 ± 7.13 µm and a higher D[4,3] of 180.56 ± 21.27 µm in comparison to ELF, which showed a higher D[3,2] of 102.98 ± 2.58 µm and lower D[4,3] of 177.22 ± 4.24 µm. Significant differences were observed in the Dv(10) and Dv(50) µm sizes whereas the Dv(90) did not.

4.3.2.19. Metabolomics

Metabolomic analysis of the ingredients showed that all of the identified compounds exhibited relatively low levels in both ingredients, as illustrated in **Supplementary Figure 4.2**. Furthermore, these compounds were found to be more depleted in ELF compared to EDF (**Figure 4.2**). Lactic acid was the most abundant metabolite found in EDF at 13.21 ± 0.79 µmol/g, and was the most significantly depleted metabolite g in ELF (data not shown in **Figure**

4.2), with levels reduced to 0.30 ± 0.04 µmol/g. Free amino acids which were abundant in EDF included lysine, phenylalanine, alanine, and leucine, whereas other metabolites and free amino acids showed values of less than 0.05 µmol/g.

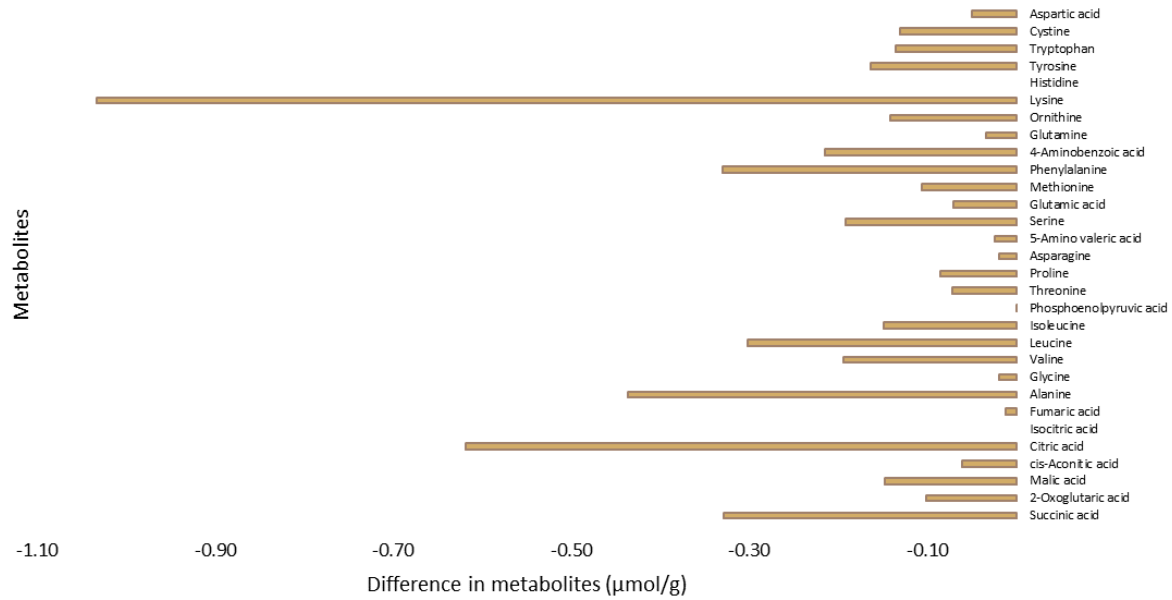

Figure 4.2: Difference in metabolites between ELF versus EDF, expressed as µmol/g.

4.3.3. Protein biochemistry

4.3.3.20. Amino acid composition and protein profile analysis

Figure 4.3A displays the % of the recommended daily essential amino acid content per gram of protein for older children (>3 years old), adolescents and adults outlined by the FAO (2013) for both EverPro ingredients. EDF showed slightly higher amounts of essential amino acids compared to ELF and therefore covers a higher % of the daily requirement**.** Based on these requirements, lysine was limited for both EDF and ELF providing only 60% and 46% of the requirement, respectively. Furthermore, both EDF and ELF exceed the requirements for the remaining essential amino acids, with the exception of ELF providing just below the histidine (90%) and leucine (98%) requirements.

The protein profiles of the ingredients under reducing conditions are shown in **Figure 4.3B.** The majority of the visible bands for both EverPro ingredients are seen between the 3.5 kDa and 15 kDa range as indicated by the increased colour density in this area, showing the presence of small peptides due to processing steps such as hydrolysis. There is also a slight indication of peptides between the 28 kDa and 46 kDa range, which could potentially be barley Protein

Z. Peptides below 3.5 kDa may also be present but cannot be identified due to the limitation of the Bioanalyzer. Overall, no difference in the protein profiles EDF and ELF was observed.

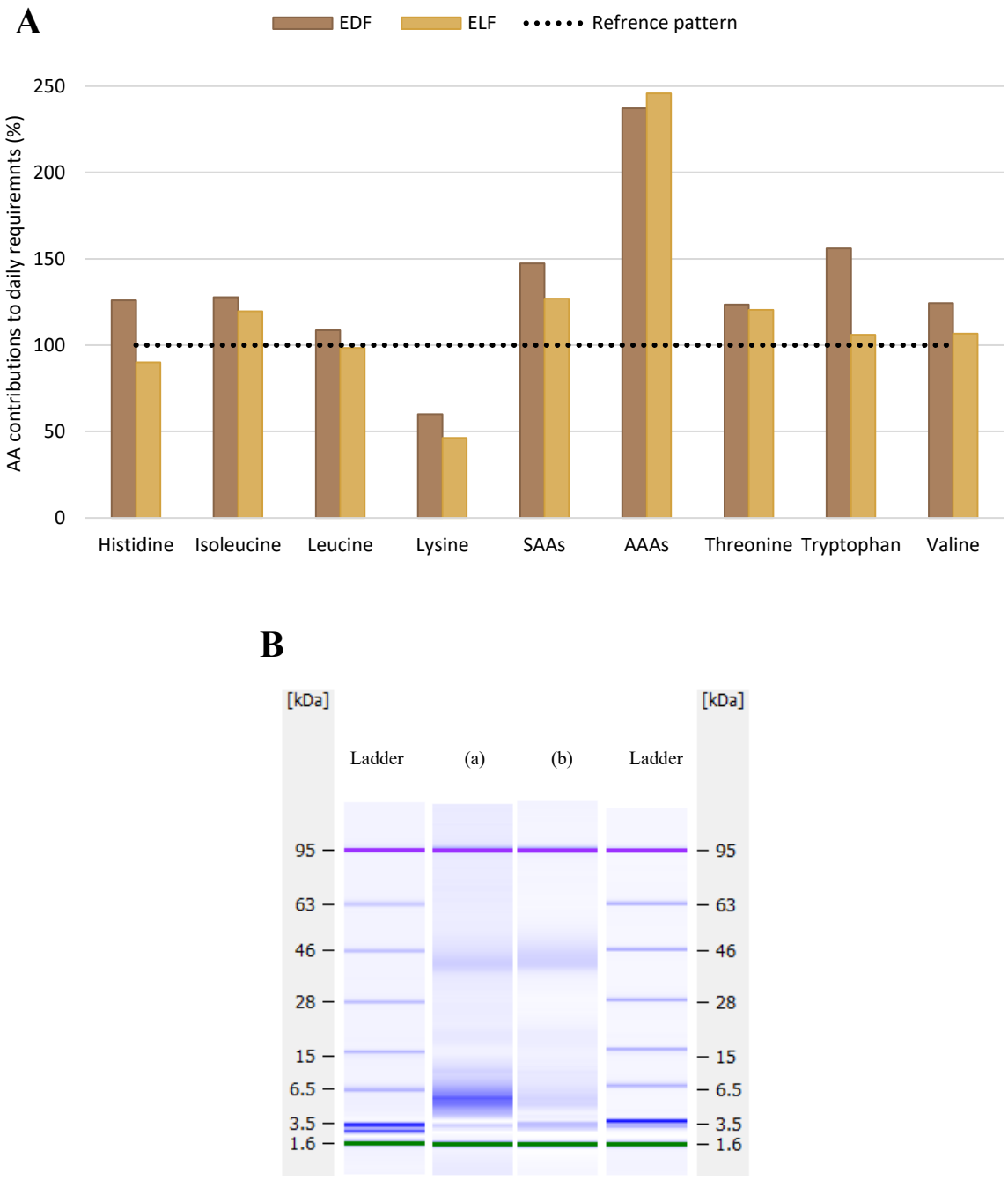

Figure 4.3: (A) Determination of the % of the daily requirement of each essential amino acid per gram of protein for older children, adolescents and adults > 3 years old outlined by FAO (2013). (B) Protein profiles of (a) EDF and (b) ELF as analysed using the Bioanalyzer under reducing conditions in the range of 5 – 80kDa.

4.3.3.21. Protein solubility and zeta potential

The protein solubility of the EverPro ingredients at pH 2, 4, 7 and 9 is presented in **Figure 4.4A**. EDF demonstrated a slightly higher solubility at pH 4, although non-significant, compared to ELF where solubility values were slightly higher at increased pH (i.e. pH 7 and 9) at a significant difference. Generally, the solubility of both EverPro ingredients was high over a broad pH range with the lowest solubility observed at pH 4, measuring at 82% ± 0.9 and 80% ± 1.2, respectively.

The zeta potential of the EverPro ingredients at pH 2, 4, 6 and 8 is shown in **Figure 4.4B**. Both EDF and ELF showed isoelectric points at approximately pH 3.4 and pH 3.3, respectively. EDF was further away from its net change at pH 8 at -39.67 ± 4.94 mV compared to ELF at -29.49 ± 6.04 mV, though this was not significantly different. At pH 4, EDF was closest to 0 at -4.66 ± 0.18 mV compared to ELF at -7.70 ± 1.24 mV at a significant difference.

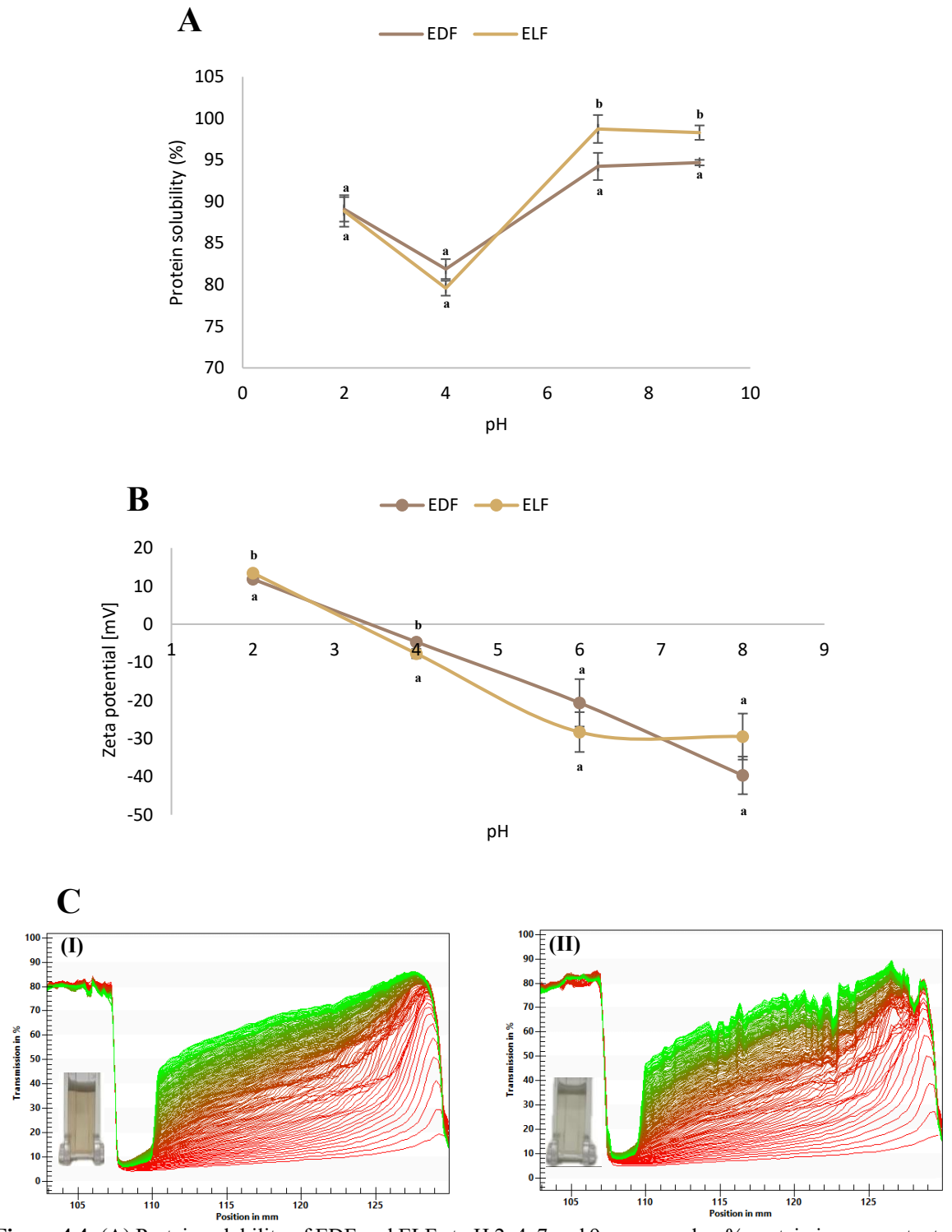

Figure 4.4: (A) Protein solubility of EDF and ELF at pH 2, 4, 7 and 9, expressed as % protein in supernatant over initial protein concentration. Values that share the same letter are not significantly different. (B) Zeta potential of EverPro ingredients at pH 2, 4, 6 and 8. Values that share the same letter are not significantly different ($p < 0.05$). (C) – Light transmission profiles of EDF (I) and ELF (II) emulsions as a function of time.

4.3.3.22. Sulfhydryl groups

Levels of exposed, free and total SH groups are shown in **Table 4.3**. Both EverPro ingredients contained a low concentration of exposed, free, and total SH groups with minor differences observed. ELF (0.187 ± 0.071) contained statistically significantly more exposed SH groups than EDF (0.065 ± 0.025) at an almost three-fold increase, however EDF had statistically significantly higher levels of free SH groups than ELF. Both ingredients contained comparable levels of total SH groups (5 - 5.2 uM/g protein) with no significant difference.

Table 4.3: Sulfhydryl groups of EDF and ELF. Values in the same row that share a letter do not differ significantly ($p < 0.05$).

	EDF	**ELF**
Exposed SH groups (µM/g protein)	0.065 ± 0.025 [a]	0.187 ± 0.071 [b]
Free SH groups (µM/g protein)	0.484 ± 0.145 [a]	0.102 ± 0.049 [b]
Total SH groups (µM/g protein)	5.068 ± 0.218 [a]	5.158 ± 0.162 [a]

4.3.4. Techno-functional properties

4.3.4.23. Emulsion characteristics

The separation rates of EDF and ELF emulsions are shown in **Table 4.2**. Both emulsions had comparable emulsifying properties. ELF separated the fastest at 4.78 ± 0.28 %/min while EDF showed a slightly slower separation rate of 4.48 ± 0.15 %/min, though the difference was non-significant. The light transmission profiles obtained by the Lumisizer and cuvette pictures (**Figure 4.4C**) showed that no sedimentation was observed in the emulsions, however creaming did occur in both emulsions.

4.3.4.24. Oil holding capacity (OHC)

OHC is the total amount of oil that can be retained by the sample. OHC of proteins are important for determining the textural properties of different food systems. As shown in **Table 4.2**. ELF (185.42 ± 7.79) displayed a significantly higher OHC than EDF (156.30 ± 10.52), an increase of approximately 20%.

4.3.4.25. Foaming capacity and foam stability

Foaming capacity and stability of protein ingredients are important characteristics in various applications for the food industry. The foaming capacity and stability of EDF and ELF are presented in **Table 4.2**. Both EDF and ELF displayed similarly high foaming capacities of 113.68 ± 6.20% and 114.63 ± 3.84%, respectively. While, the foam stability of both ingredients

was low, EDF showed significantly higher foam stability at 35.31 ± 0.84% compared to ELF at 15.17 ± 1.31%.

4.3.5. Sensory attributes and tribology

4.3.5.26. Aroma profile of powders

Overall, 59 active aroma compounds were identified during the GC-O analysis. 53 odour-active compounds were identified for EDF, while 37 odour-active compounds were detected in ELF, and the intensity of each compound can be seen in **Supplementary Table 4.1**. For simplicity, the difference in the intensities of the single aromas detected in ELF versus EDF are displayed in **Figure 4.5A**. Overall, the processing of EDF into ELF resulted in the decrease of 14 compounds, and elimination of 20 compounds (total of 34 compounds), including compounds associated with butter, cabbage, earthy, boiled potato, cress, meaty, aniseed, hay, fenchol, roasty, fatty, caramel, seasoning, clove, ham, peach, fecal, soapy, and wax aromas. Specific Maillard reaction products, such as the aldehydes 2-methylpropanol (malty 1), 2- and 3-methylbutanal (malty 2), and hexanal (grassy), were lower in concentration in ELF. Additionally, other aroma-active compounds, such as (Z)-4-heptenal (fishy), (E,Z)-2,6-nonadienal (cucumber), 2-ethyl-3,5-dimethylpyrazine (earthy 1) and 2-methoxy-4-vinylphenol (clove), were also lower or even absent in ELF. Compounds with 'meaty' descriptors that were depleted in the processing of EDF to ELF included 2-methyl-3-(methyldithio)-furan (meaty 1), 2-methyl-3-(methylthio)-furan (meaty 2), and 2-ethyl-3-mercapto-1-hexanol (meaty 3). Conversely, several compounds described as malty, roasty, marzipan, almond, floral, metallic, seasoning, foxy, peach, and cheese had higher intensity in ELF. Representatives of these compounds are 2-acetyl-1-pyrroline (roasty 1) and 1-octen-3-one (mushroom).

The expert sensory panel characterised EDF as having more pronounced malty, seasoning, smoky, caramel, and earthy roasty odour notes, with weaker fatty and plastic notes (**Figure 4.5B**). On the contrary, ELF was characterized more by fatty notes but with less malty, seasoning, smoky, caramel and earthy/roasty odour. Not-withstanding this, comments from the sensory panellists indicated that the overall odour intensity of both EDF and ELF was weak.

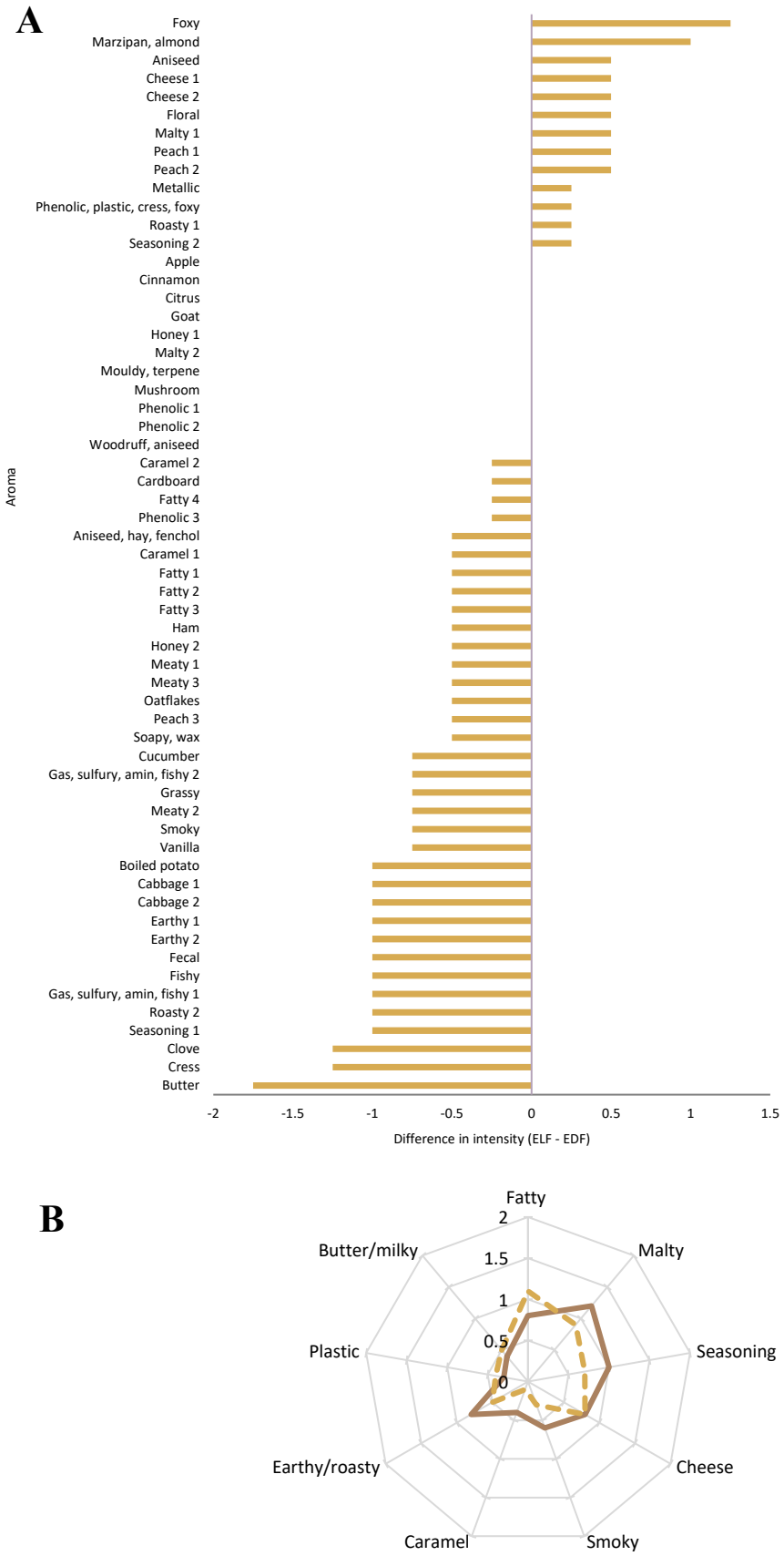

Figure 4.5: (A) Gas chromatography—olfactory analysis representing the difference between ELF versus EDF in odour intensity of aroma compounds. (B) Sensory panel aroma characteristics of EDF (solid line) and ELF (dashed line) and their associated intensities.

4.3.5.27. Descriptive sensory analysis

Sensory analysis showed perceived significant differences between EDF and ELF (**Table 4.4**). ELF was perceived to have lower bitterness and lower burnt/roasty flavour compared to EDF. ELF was also perceived to have a more viscous mouthfeel at 6.88 ± 1.15 compared to EDF at 5.79 ± 1.86. All others sensory attributes were perceived to be similar between EDF and ELF with no significant differences found.

Table 4.4: Results of the sensory analysis of EDF and ELF. Values in the same row that share a letter do not differ significantly ($p < 0.05$). Descriptors for sensory attributes shown in **Supplementary Table 4.2**.

	EDF	**ELF**
Taste: Bitter	6.46 ± 2.78 [a]	3.92 ± 2.47 [b]
Taste: Salty	3.96 ± 2.39 [a]	3.96 ± 2.10 [a]
Taste: Umami	3.33 ± 1.93 [a]	3.79 ± 2.54 [a]
Flavour: Burnt/roasty	7.38 ± 2.36 [a]	4.29 ± 2.37 [b]
Flavour: Caramel	6.38 ± 2.65 [a]	1.75 ± 1.51 [a]
Flavour: Soapy	2.08 ± 1.47 [a]	4.96 ± 2.69 [a]
Flavour: Earthy	4.67 ± 3.52 [a]	2.92 ± 2.52 [a]
Flavour: Malty/cereal	3.83 ± 2.99 [a]	5.00 ± 2.72 [a]
Mouthfeel: Viscosity	5.79 ± 1.86 [a]	6.88 ± 1.15 [b]

4.3.5.28. Tribology

Soft tribology is a technique used to measure the frictional properties of food and beverages which can be linked with mouthfeel. **Figure 4.6** displays the Stribeck curves of both EDF and ELF compared to water. The presented curves for the ingredients can be divided into static (< 10-8 – 10-5 m/s), boundary (10-5 – 10-4 m/s) and beginning of mixed (> 10-4 m/s) regimes. 10% (w/w) solutions of both samples showed a substantial increase in lubrication properties compared to water. ELF exhibits lower friction factors in comparison to EDF, suggesting that ELF possesses higher lubricating properties. This increase in lubrication was seen to be significant at the sliding speed 10-5 m/s, with friction factors of 0.1997 ± 0.0201 for EDF and 0.1387 ± 0.0315 for ELF and at the sliding speed 10-1 m/s, with friction factors of 0.0237 ± 0.0039 for EDF and 0.0181 ± 0.0011 for ELF.

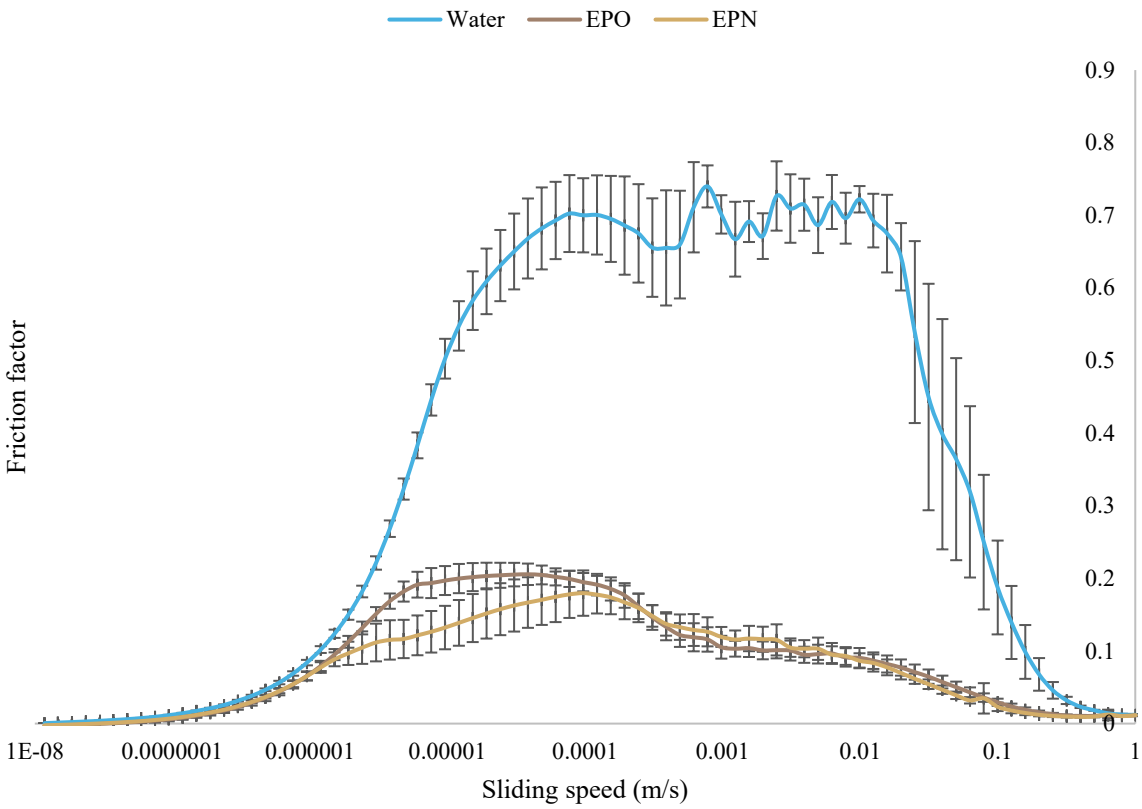

Figure 4.6: Stribeck curves of water, EPO and EPN solutions (10% w/w) with friction factor as a function of sliding speed (m/s).

4.4. Discussion

Barley and rice protein isolate upcycled from BSG is gaining popularity as a novel plant-based protein ingredient. Moreover, as a by-product from the brewing process, specifically after the malting and mashing steps, the utilisation of this side stream represents a sustainable source of protein (Lynch et al., 2016). This study investigated the nutritional and techno-functional properties of two barley and rice protein isolates EverPro® Dark Fraction (EDF) and EverPro® Light Fraction (ELF). Exploring the functional characteristics of ELF, which underwent further processing, and comparing it with EDF is crucial for assessing its quality and potential suitability for application into additional food and beverage matrices.

The nutritional composition of EDF and ELF is comparable. With regards to the key macronutrient, protein, the content in each ingredient exceeds 80%, with ELF showing a slightly higher amount than EDF, likely attributed to the additional processing. The criteria to assess the quality of a protein ingredient is its amino acid composition. Both these ingredients contain a high quantity of branched chain amino acids (BCAAs), which are valuable for athletes seeking to reduce exercise recovery time and for elderly to enhance muscle mass (Shimomura et al., 2004). With the exception of lysine, all essential amino acids in EDF surpassed the requirements per gram of protein set by the FAO, (2013) guidelines for children > 3 years old, adolescents and adults. Lysine has been identified as the limiting amino acid for cereal-based proteins. A recent study by Jaeger et al. (2024) revealed that the *in vitro* digestible indispensable amino acid score (DIAAS) of EDF at 67%, surpassed other plant proteins including a pea, soy and brown rice protein isolate. The DIAAS gives an indication of protein quality in relation to its bioavailability (Mathai et al., 2017). A balanced combination of cereal and legume proteins may provide a higher quality protein blend, presenting a complete essential amino acid profile and increased DIAAS (Gorissen et al., 2018). Many commercial vegan products on the market, such as plant-based milks, yogurts, cheeses, eggs, and burgers often fail to provide the same protein content and quality as their animal-based counterparts (Boukid & Castellari, 2021; Craig et al., 2022; Craig & Brothers, 2021; Halm et al., 2024; Jeske et al., 2017). The incorporation of proteins like EverPro into such products can improve the protein value and assist plant-based products to achieve nutritional comparability to their animal counterparts with respect to protein quality.

Whey protein isolate and sodium caseinate are common animal-based protein ingredients that possess a whiter colour compared to most plant-based proteins like pea, soy, lentil, chickpea

and quinoa isolates which tend to possess yellow, green, or red to brown colours. This can limit their application in certain products (Arteaga et al., 2021; Steffolani et al., 2016; Tang et al., 2023). Studies have reported that BSG-derived ingredients typically exhibit low L*-values, resulting in a dark coloured ingredient. Additionally, it has been shown that a greater degree of hydrolysis of BSG to extract the protein resulted in lower L*-values (Celus et al., 2007; Jaeger et al., 2023, 2024). Several factors during the brewing process contribute to the colour formation of BRPI, including the Maillard reaction which produces brown coloured compounds like melanoidin, as well as the protein extraction process which potentially results in EDF possessing a dark brown colour (Coghe et al., 2006; Zhao, 2014). This dark colour characteristic can make the ingredient less applicable in certain products and limit its flavour profile by the impact of colour on flavour perception (Sakai et al., 2022; Spence, 2015). However, further processing of EDF into ELF resulted in a colour change towards a more neutral sandy/beige colour. Enhancing ingredient utility through additional processing is common practice in the food industry. One method industry applies includes a bleaching step that removes colour to achieve a whiter appearance. Bleaching can be achieved through enzymatic means or by the addition of a chemical oxidizing agent often employed using chlorine gas/dioxide or benzoyl/hydrogen peroxide (Kang et al., 2010). Regulations for bleaching flours in the US are defined by the Food and Drug Administration under cereal flours and related products 137.105 where flour bleaching in the EU is prohibited (European Parliament & Council, 2008; FDA, 2023). In the EU, flour is described as the basic milled products of vegetable commodities such as cereal grains, roots, tubers, pulses, soybeans and potatoes with the exception of starches. Since plant-based proteins are the protein components within the vegetable commodity, this implies they are also prohibited from being bleached (European Parliament & Council, 2008). Occasionally in the US, flour is bleached in order to achieve a whiter colour due to the presence of carotenoid pigments, as is bleaching whey protein isolate when annatto is added during the cheese-making process (Jervis et al., 2012; Saunders et al., 2007). Moreover, it has been demonstrated that bleaching can enhance the functionality of both wheat flour and soy protein isolate, although limited studies have been completed, (Sakai et al., 2022; Van Haesendonck et al., 2018). The decolourisation of certain plant-based protein isolates, that exhibit yellow to brown colours, in some cases is necessary to enhance their physical properties where colour is the first aspect of food products noticed by consumers and plays a significant role in their perception of taste and overall product acceptance (Sakai et al., 2022; Spence, 2015). Hence, whitening the ingredient becomes crucial for aesthetics in product applications.

Protein solubility and zeta potential are often positively correlated with one another. Protein solubility is an important property when investigating suitable applications for plant-based ingredients, with a high solubility desirable for applications in liquid food systems, such as beverages. Both EDF and ELF are highly soluble over a range of pH values, especially at higher pH values. ELF achieved significantly higher solubility at pH 7 and 9 compared to EDF, similar to finding in decolourised soy protein isolate treated with hydrogen peroxide as a bleaching reagent. This increase in solubility was attributed to the removal of phenolic compounds, formation of covalent crosslinking and/or expansion of protein tertiary structure, suggesting similar mechanisms may have occurred in the decolourisation process of EDF to ELF (Sakai et al., 2022). A slight decrease in solubility was observed at acidic pH values, particularly at pH 4. This trend has been previously reported for BSG protein isolates, where a higher solubility has been attributed to a higher degree of hydrolysis of ingredients, causing a decrease in molecular weight, unfolding of the protein structure, and increased ionic strength i.e. repulsive forces (Celus et al., 2007; Connolly et al., 2014; Wouters et al., 2016; Yalçin & Çelik, 2007). The presence of low molecular weight peptides in EDF and ELF was evident in the protein profile analysis, aligning with previously reported results for EDF (Jaeger et al., 2024). The presence of a protein with a molecular weight between 28 kDa and 46 kDa most likely indicates the presence of protein Z, a major barley albumin with a molecular mass of approximately 40 kDa that has been shown to be heat stable and resistant to enzymatic degradation (Jaeger et al., 2021). Additionally, protein Z is a human vitamin K dependent coagulation protein that acts as a cofactor for regulating blood clotting (Almawi et al., 2013). Hence, even further treatment of EDF did not change the protein profile. Solubility of EDF and ELF was lowest at pH 4 revealing the proximity of the isoelectric point whereby protein aggregation may occur, potentially resulting in increased turbidity and protein sedimentation if the ingredient is applied in a beverage system in this pH range. Attaining a solubility higher than 80% at acidic pH ranges is uncommon for plant-based proteins, with values of less than 30% commonly observed at pH 4-6 for ingredients with an isoelectric point within this range (Ma et al., 2022; Tang et al., 2023). Thus, EDF and ELF are unique with respect to their high solubility at acidic pH. Zeta potential serves as a critical factor in assessing the dispersion of particles and predicting stability, providing insights into the electrostatic repulsive forces and surface charge. The zeta potential threshold values which differentiate stable and unstable systems are typically considered within the ranges of ± 0-10 mV (very stable), ± 10-20 mV (relatively stable), ± 20-30 mV (moderately stable), and ± 30 mV (highly stable). Regarding proteins, the pH, ionic force and temperature highly influences surface charge and impacts

aggregation (Cano-Sarmiento et al., 2018). In the current study, EDF and ELF exhibited greater stability at the higher pH levels approaching a zeta potential closer to ± 30 mV. In contrast, they were least stable at acidic pH values. Typically, soluble complexes arise when electrostatic interactions are weak and net charge is high, whereas insolubility occurs when electrostatic interactions are stronger, and the net charge is low (Cano-Sarmiento et al., 2018). These results link with protein solubility, both indicating an isoelectric point at pH 4.

Particle size can also influence the stability of a colloidal system (Chu et al., 1996), however, when looking at the D[4,3] particle size, further processing of EDF to produce ELF had no significant effect. Analytical centrifugation for accelerated stability analysis is a rapid and effective way for predicting gravitational separation patterns. Common instability events in emulsions include gravitational separation due to upward creaming or downward sedimentation movement of droplets (Grossmann & McClements, 2023). EDF and ELF displayed similar emulsifying abilities, with upward movement of particles in both emulsions, evidenced as the formation of a cream layer on the surface of the emulsion, and visible in the transmission profiles. Similar emulsifying ability has been previously reported for EDF (Jaeger et al., 2023), meaning further processing of EDF to ELF did not affect emulsifying properties.

OHC can be associated with the sensory quality of food including aspects such as mouthfeel and flavour retention. OHC of both ingredients was high, with ELF showing directional ability to retain slightly more oil than EDF, but this result was not significant. Sulfhydryl groups can play a significant influence on the gel forming networks of a given protein ingredient (Havea et al., 2009; Hu et al., 2013). EDF and ELF contained low amounts of sulfhydryl groups potentially influenced by the degree of hydrolysis. Hence it could be predicted that the gel strength of both EDF and ELF would demonstrate poor stability. Additionally, protein hydrolysis has been shown to improve foaming capacity of proteins by facilitating their diffusion to and adsorption at the interface. However, hydrolysis can negatively impact foam stability due to the decrease in molecular mass of the protein (Celus et al., 2007; Wouters et al., 2016). This has was observed in both EDF and ELF. The lower foam stability in ELF might be due to less exposed SH-groups, as protein-based foams are reliant on molecular size, conformation, hydrophobicity, and solubility of the proteins that can allow increase in protein flexibility where the protein is then able to diffuse to the air – water interface to encapsule air molecules (Kinsella, 1981).

Changes in the techno-functionality of ELF due to further processing were marginal. However, significant alteration in perceived sensory attributes did occur. The vast majority of identified volatile compounds are associated with malted barley (Almaguer et al., 2023), and were notably lower in ELF compared to EDF. Lower perceived burnt/roasty and earthy flavours in ELF is most likely due to the reduction in Maillard flavour compounds, such as acetyl-2-thiazoline, 3-methoxy-2,5-dimethylpyrazine and 2-ethyl-3,5-dimethylpyrazine. The sensory assessments of the trained panel were supported by the GC-O measurements. The presence of less Maillard reaction products resulted in lower perceived burnt/roasty, and earthy flavours. Burnt/Roasty flavours and aromas are often associated with bitterness (Münchow et al., 2020).

Even though further processing of proteins has a high potential of forming bitterness and/or off-flavours (Kato et al., 1989; Wouters et al., 2016), ELF was perceived to be significant less bitter compared to EDF. Phenolic compounds are often associated with bitter flavours (Drewnowski & Gomez-Carneros, 2000), and further processing of EDF may have caused elimination of some bitter polyphenols. The slight increase in umami taste in ELF is most likely due to an increase in 2-/3-methylbutanoic acid which is present in fermented meat products (Chen et al., 2024). Studies have shown that the decolourisation of whey by chemical means or enzymatic treatment can increase lipid oxidation resulting in a higher intensity of carboard and fatty flavours (Carter & Drake, 2018). This, however, was not the case after the decolourisation of EDF to produce ELF.

The sensory panel judged ELF as having higher perceived mouthfeel compared to EDF. To investigate this further, tribology was performed on 10% solutions of EDF and ELF. This method is emerging as an instrumental tool to characterise mouthfeel/texture, with studies already performed on beverages (Newtonian fluids) like beer, wine, tea and soft drinks (Chong et al., 2019; Fox et al., 2022; Laguna & Sarkar, 2017; Steinbach et al., 2014). In general, a higher friction factor can indicate increased surface roughness with lower friction having the opposite effect, referring to changes in lubrication properties (tribological behaviour). In order to analyse sensory attributes of liquids using tribology, fluid dynamics of the relevant samples play a key role. Dispersions of EDF and ELF both behave like Newtonian fluids (Jaeger et al., 2023), meaning that under shearing conditions (with constant temperature and pressure), shear stress is proportional to shear rate with no changes in dynamic viscosity (Paul et al., 2022). Also, both EDF and ELF displayed lower friction compared to water due to their surface-active properties like other plant proteins (Vlădescu et al., 2023). ELF displayed lower friction compared to EDF. There are two main factors that influence lubrication of a dispersion; first,

the particle size which in the case of EDF and ELF can be neglected since no significant differences were observed, and second, the particle shape. The morphology of EDF and ELF is very similar; however, EDF showed a rougher surface which could cause increased friction. ELF, on the other hand, has a smooth cover on the surface which, likely contributed to lubrication (see SEM micrographs). Moreover, the degree of hydrolysis of proteins and the presence of free amino acids and organic acids can impact friction. Higher molecular weight protein can form a thicker film and thus increase friction (Upadhyay et al., 2016). As hypothesised above, the further processing of EDF to ELF can have caused further protein hydrolysis and thus enhanced lubrication. In food products friction is linked with the perception of astringency and bitterness (Upadhyay et al., 2016). Interestingly, the sensory panel evaluated EDF, which had a higher friction than ELF, as being more bitter in taste. Metabolic compounds, specifically organic acids, are reported to increase astringency in beverages (Laguna & Sarkar, 2017), and hence friction. The reduced concentration of metabolites in ELF compared to EDF, particularly lactic acid, may have also contributed to the lower friction ELF solution.

4.5. Conclusion

BRPI is a novel plant-based protein sourced from BSG, a brewing by-product. As well as offering a source of high quality plant-based protein, EDF and ELF offer a more sustainable source of protein, especially with sustainability now at the forefront of product development. Both ingredients have the potential to increase the protein content and nutritional value of a food or beverage product. The following study revealed that EDF and ELF were comparable with respect to nutritional value and several techno-functional properties. Key differences observed for ELF included colour, taste and flavour attributes. Compared to EDF, ELF has a higher brightness that can increase its utility across a wide variety of food and beverage products. Brightness can enhance visual appeal and facilitate better consumer acceptance. Moreover, ELF contained a reduced amount of metabolites and aroma compounds associated with reduced bitterness and burnt/roasty notes in comparison to EDF, providing a more ‘neutral’ flavour. Difference in sensory profile were supported by differences in tribology with ELF demonstrating higher lubrication properties than EDF at certain sliding speeds. That supports the lower bitterness and higher viscosity perceived by a trained sensory panel. Further research should investigate the application of these novel ingredients into a variety of food products, with beverage systems of particular interest given the high solubility of both protein

ingredients. Therefore, EverPro® ingredients are potential candidates for a variety of beverage products seeking added protein value including sports drinks and/or functional beverages.

4.6. Acknowledgements

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4.7. Supplementary Material

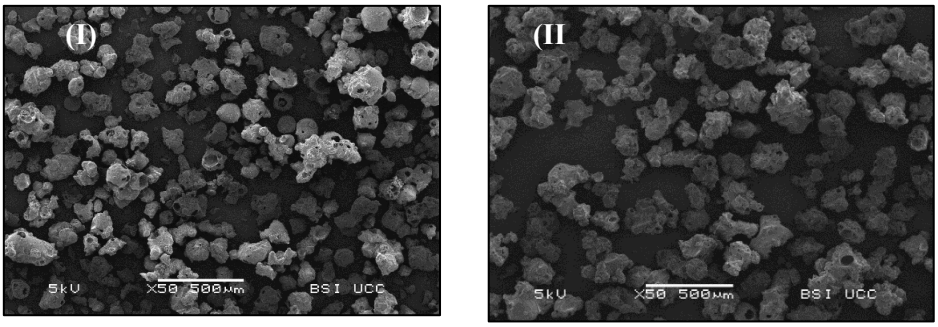
Supplementary Table 4.1: Gas chromatography—olfactory analysis representing the odour intensity of selected aroma compounds; 0 = not detected, 1 = weakly detected, 2 = unequivocally detectable, 3 = intensely detectable.

Compound	**Aroma**	**Intensity**		
		EDF	***ELF***	***Difference in intensity***
2-aminoacetophenone	Foxy	0.75	2	1.25
Benzaldehyde	Marzipan, almond	0	1	1
2-/3-methylbutanoic acid	Cheese 2	1	1.5	0.5
3-methyl-2,4-nonandione	Floral	0.5	1	0.5
Y-nonalactone	Peach 1	0.5	1	0.5
p-anisaldheyde	Aniseed	0	0.5	0.5
Butanoic acid	Cheese 1	0	0.5	0.5
2-methylpropanal	Malty 1	0	0.5	0.5
Y-decalactone	Peach 2	0	0.5	0.5
2-acetyl-1-pyrroline	Roasty 1	1.5	1.75	0.25
Trans-4,5-EDFxy-(E)-2-decenal	Metallic	1	1.25	0.25
Unknown	Phenolic, plastic, cress, foxy	0.75	1	0.25
Sotolon	Seasoning 2	0.5	0.75	0.25
2-/3-methylbutanal	Malty 2	2.25	2.25	0
1-octen-3-one	Mushroom	1.5	1.5	0
Octanal	Citrus	1	1	0
3-/4-methylphenol	Phenolic 1	1	1	0
(E)-β-damascone/ E)-β-damascenone	Apple	0.5	0.5	0
(E)-ethylicinnamate	Cinnamon	0.5	0.5	0
phenylpropanoic	Goat	0.5	0.5	0
2-phenylethanol	Honey 1	0.5	0.5	0
Unknown	Mouldy, terpene	0.5	0.5	0
3-/4-propylphenol	Phenolic 2	0.5	0.5	0
Unknown	Woodruff, aniseed	0	0	0
(E)-nonenal	Cardboard	1.5	1.25	-0.25
(E,E)-2,4-decadienal	Fatty 4	1	0.75	-0.25
Furaneol	Caramel 2	0.75	0.5	-0.25
3-/4-propylphenol	Phenolic 3	0.75	0.5	-0.25
Phenylacetic acid	Honey 2	1.25	0.75	-0.5
(E,E)-2-4-nonadienal	Fatty 2	1	0.5	-0.5
(E,E,Z)-2,4,6-nonatrienal	Oatflakes	1	0.5	-0.5
Unknown	Aniseed, hay, fenchol	0.5	0	-0.5
Maltol	Caramel 1	0.5	0	-0.5
(Z)-2-decenal/(E,E)-2,4-octadienal	Fatty 1	0.5	0	-0.5
(E,Z)-2,4-decadienal	Fatty 3	0.5	0	-0.5
Isoeugenol	Ham	0.5	0	-0.5
2-methyl-3-(methyldithio)furan	Meaty 1	0.5	0	-0.5
2-ethyl-3-mercapto-1-hexanol	Meaty 3	0.5	0	-0.5

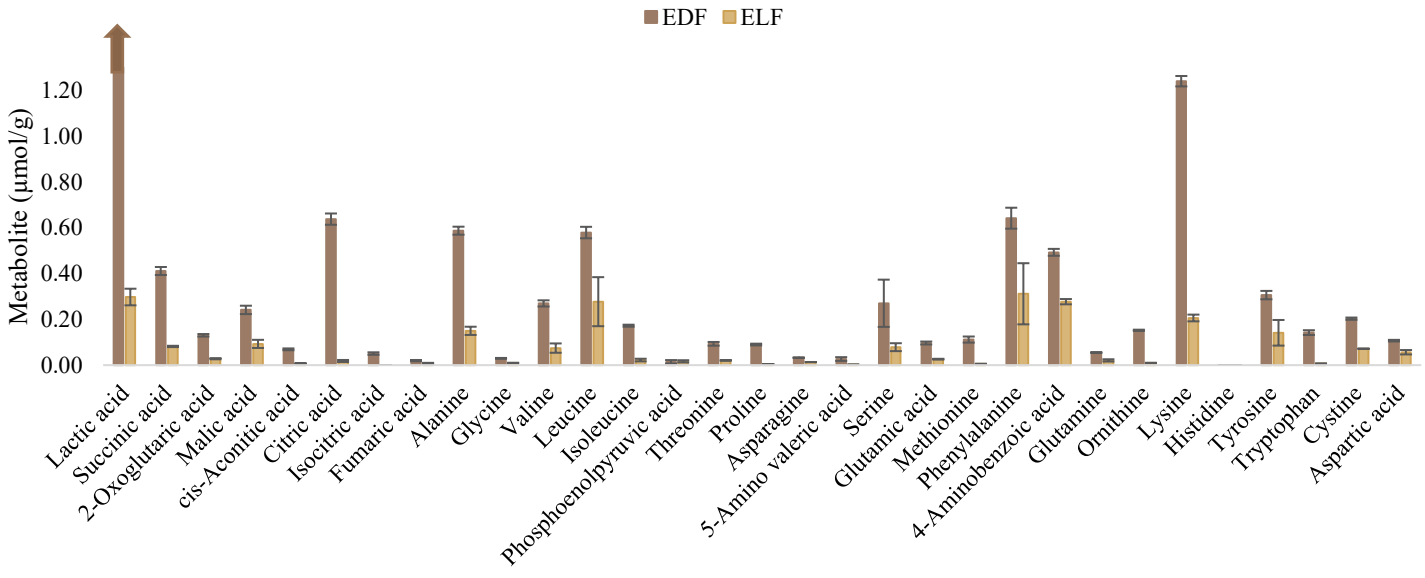
Y-dodecalactone	Peach 3	0.5	0	-0.5
Unknown	Soapy, wax	0.5	0	-0.5
Vanillin	Vanilla	1.5	0.75	-0.75
(E,Z)-2,6-nonadienal	Cucumber	1.25	0.5	-0.75
Unknown	Gas, sulfury, amin, fishy 2	1.25	0.5	-0.75
Hexanal	Grassy	1.25	0.5	-0.75
2-methoxyphenol	Smoky	1.25	0.5	-0.75
2-methyl-3-(methylthio)furan	Meaty 2	0.75	0	-0.75
1-penten-3-ol	Gas, sulfury, amin, fishy 1	2.25	1.25	-1
(Z)-4-heptenal	Fishy	2	1	-1
Methional	Boiled potato	1	0	-1
Dimethyltrisulfide	Cabbage 1	1	0	-1
Dimethyltetrasulfide	Cabbage 2	1	0	-1
3-methoxy-2,5-dimethylpyrazine	Earthy 1	1	0	-1
2-ethyl-3,5-dimethylpyrazine	Earthy 2	1	0	-1
3-methylindol	Fecal	1	0	-1
2-acetyl-2-thiazoline	Roasty 2	1	0	-1
Unknown	Seasoning 1	1	0	-1
2-methoxy-4-vinylphenol	Clove	1.25	0	-1.25
Benzylmercaptane	Cress	1.25	0	-1.25
2,3-butanedione	Butter	1.75	0	-1.75

Supplementary Table 4.2: Descriptors for sensory evaluation attributes.

Attribute	*Description*	*Intensity*
Taste		
Bitter	Perceived bitterness	0 = none, 10 = extreme
Salty	Perceived saltiness	0 = none, 10 = extreme
Umami	Perceived savoury or meaty taste	0 = none, 10 = extreme
Flavour		
Burnt/Roasted	Perceived burnt and roasty flavour	0 = none, 10 = extreme
Caramel	Toasted sugar and toffee-like	0 = none, 10 = extreme
Soapy	Detergent-like, chemically	0 = none, 10 = extreme
Earthy	Woody, musky	0 = none, 10 = extreme
Malty/Cereal	Bread/biscuit-like	0 = none, 10 = extreme
Mouthfeel		
Viscosity	Perceived thickness or thinness of the sample, smoothness on the palate	0 = none, 10 = extreme



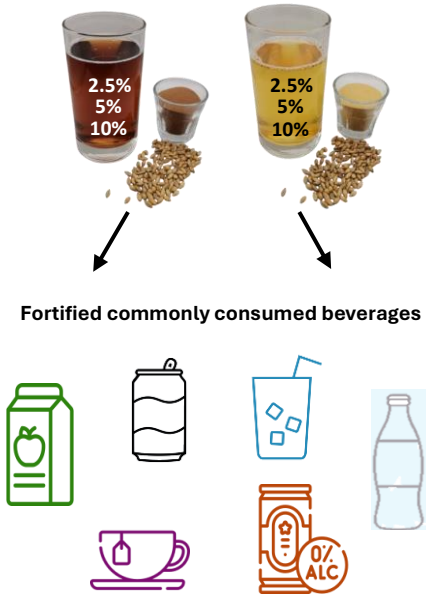
Supplementary Figure 4.1: Scanning electron microscope (SEM) imaging of EDF (I) and ELF (II) at 50× magnification.



Supplementary Figure 4.2: Metabolites in EDF and ELF, expressed as µmol/g.

5. Chapter 5

Benefits and challenges of fortified beverage systems with brewer's spent grain-derived protein isolates



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Abstract

Plant protein soft drinks align with current consumer demand for hydration, nutrition and sustainability yet faces challenges due to protein aggregation and precipitation in an acidic environment. This study evaluated the effects of two different barley and rice protein isolates, EverPro® Dark Fraction (EDF) and a decolourised version, EverPro® Light Fraction (ELF), at ingredient inclusion levels of 0%, 2.5%, 5% and 10% in diverse beverage systems including water as a control, apple juice, a full sugar and sugar free carbonated drink, a sweetened ice tea, an unsweetened black tea and a non-alcoholic beer. Physicochemical properties were comprehensively assessed. Protein inclusion, particularly at 10%, elevated pH to a more neutral range (from 3.1 and 4.88 to 5.0 and 7.8). Increasing the addition of both ingredients also elevated, viscosity (from 1.04 and 1.46 to 2.54 and 5.43 mPa·s), density (0.999 and 1.045 to 1.030 and 1.074 g/cm^3), as well as lubrication properties of all beverages with ELF exhibiting higher values compared to EDF. Dispersion stability improved dramatically including turbidity, and particle size at higher inclusion levels (10%), influenced by changes in pH further from the proteins isoelectric point. Low inclusion levels (2.5%) caused maximum turbidity and separation rate. Principal component analysis revealed distinct clustering by beverage matrix and protein type, with apple juice most affected. These finding demonstrate a step towards soft drinks fortified with upcycled protein from brewers spent grain with added nutritional benefits for future commercially potential.

5.1. Introduction

Conventional soft drinks offer little to no nutritional value, primarily composed of water, sugar or sweeteners, acidulants and flavourings (Ashurst et al., 2017). Other soft drinks, such as juices, teas and non-alcoholic beers, also offer limited nutritional value but can provide essential micronutrients including vitamins, minerals, antioxidants and polyphenols. Protein-based beverages are continuing to grow as popular functional or sports products. Most research has been focused on plant-based milk alternatives and the ongoing challenges they face, such as nutrition, stability and flavour (Xie et al., 2023). These plant-based beverages or drinks are made from a variety of plant sources including cereals, nuts, and legumes, whereas typical commercial plant-based milk substitutes include the use of almonds, cashews, hazelnuts, rice, quinoa, oat, hemp and soy (Giugliano et al., 2023; Jeske et al., 2017; Mäkinen et al., 2016). Consumers are seeking a shift away from traditional protein smoothies or shakes to clear beverage options that can address thirst and provide hydration (Gupta et al., 2023). Beverages such as these can be a convenient option to supply a high dose of protein. The functional drinks market is one of the fastest growing industries, with functional waters emerging as one of the most popular beverages in this category (Gupta et al., 2023). However, most soft drinks have an acidic pH range, which can cause proteins to precipitate (Pelegrine & Gasparetto, 2005). Furthermore, these beverages are commonly thermally processed to extend their shelf life by reducing harmful microbes and inactivating enzymes. This is typically achieved through methods like pasteurisation or ultra-high temperature (UHT) sterilisation, which may alter their flavour, aroma and colour (Giugliano et al., 2023; Mäkinen et al., 2016). As a result, stabilising protein in both acidic conditions and high temperatures presents a challenge, as turbidity, phase separation, viscosity and gelation can be affected (Ryan et al., 2012). Additionally, the incorporation of plant-based proteins into beverages is often associated with challenges such as sensorial, imbalanced amino acid profiles, low bioavailability, and the presence of antinutrients. These sensory challenges can include undesirable beany flavours, astringency, bitterness and mouthcoating mainly attributed from the polyphenols(Bessada et al., 2019; Gorissen et al., 2018; Ma et al., 2022). These hurdles can limit consumer acceptance. Specifically for carbonated beverages, including high levels of protein could also lead to processing issues during production such as excessive foaming (Ashurst et al., 2017; Cermeño et al., 2024; Tang et al., 2023). Protein hydrolysates can be an excellent ingredient for fortifying these beverages due to their high solubility and greater thermal and acidic stability when compared to larger molecular size proteins. Methods used for hydrolysing proteins can include

biological processes (including fermentation and enzyme treatment), physical, or chemical processes, while each method has its own advantages and disadvantages (Ashaolu, 2020). Brewing is an example of a process that causes protein degradation leading to partially hydrolysed proteins and small peptides in brewers spent grain (Jaeger et al., 2024).

The demand for high protein food and beverage products has risen significantly in recent years with a particular emphasis on plant-based protein options as consumers increasingly identify as vegetarian, vegan or even flexitarian, and due to environmental and ethical considerations (Henchion et al., 2017). As a result, majority of the leading dairy companies are incorporating plant-based alternatives into their product portfolio. Numerous studies have focused on animal-based protein beverages, particularly fortifying various flavoured soft drinks with whey protein (Djurić et al., 2004; Goudarzi et al., 2015; Koffi et al., 2005; Yadav et al., 2016). Many of these whey-based flavoured soft drinks are formulated with low to medium protein contents (ranging from 0.8% to 6% per 100 ml) and are limited to certain beverage types such as juices, while tea and non-alcoholic beers have not been explored. Moreover, there has been limited research on the inclusion of plant-based proteins in soft drinks.

The valorisation of protein ingredients recovered from food processing side streams is critical to reduce food waste and improve resource efficiency for producing alternative proteins to address future protein demands. EverPro® Dark Fraction (EDF) and Everpro Light Fraction (ELF) produced by the company EverGrain Ingredients, are commercially available barley and rice protein isolates derived from upcycled brewers spent grain (Lynch et al., 2016; Turck et al., 2023). ELF (beige/sandy colour) is a decolourised version of EDF (dark brown colour), with higher L* brightness values and distinctive physiochemical properties (Ahern et al., 2025).

This study tested two hypotheses 1) Does EDF and ELF affect techno-functionality of the beverages? And if so, is the impact similar or significantly different? and 2) Does the ingredient addition level change the techno-functionality of the beverages? If yes, are these changes beneficial or do they cause challenges?

5.2. Materials and methods

5.2.1. Materials

The barley and rice protein isolates, EDF and ELF were obtained from EverGrain Ingredients (St. Louis, MO, USA). The apple juice from concentrate (AJ), carbonated lemon and lime soft drink (LL), carbonated lemon and lime soft drink sugar free (LL Free), sweetened peach flavoured ice tea (IT), unsweetened black tea bags (BT) and non-alcoholic lager beer (NAB) were all purchased from local supermarkets in Cork, Ireland. The list of ingredients and nutritional composition of each beverage used in the study are shown in **Table 5.1**. Chemicals for the analyses were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Table 5.1: Ingredients list and nutritional composition in grams per 100ml of beverages from labels. An * - indicates carbonated soft drink.

Soft Drink	Calories (kcal)	Carbohydrates (g)	Of which sugar (g)	Ingredients List
Water	0	0	0	Water
Apple Juice	46	11.1	11.1	Apple juice from concentrate
Lemon & Lime*	19	4.7	4.7	Carbonated Water, Sugar, Acids (Citric Acid, Malic Acid), Natural Lemon and Lime Flavouring with other Natural Flavourings, Acidity Regulator (Sodium Citrate), Sweeteners (Acesulfame K, Sucralose)
Lemon & Lime Free*	1	0	0	Carbonated Water, Acids (Citric Acid, Malic Acid), Natural Lemon and Lime Flavouring, Acidity Regulator (Sodium Citrate), Sweeteners (Aspartame, Acesulfame K), Preservative (Sodium Benzoate)
Ice Tea Peach	13	3.1	3	Water, Sugars (Sucrose, Fructose), Acids (Malic Acid, Citric Acid), Black Tea Extract (0.12%), Peach Juice from Concentrate (0.1%), Acidity Regulator (Trisodium Citrate), Flavourings, Antioxidant (Ascorbic Acid), Sweetener (Steviol Glycosides from Stevia)
Black Tea	0	0	0	100% Black Tea
Non – Alcoholic Beer*	14	3.1	0.2	Water, Malted Barley, Hops, Yeast

5.2.2. Preparation of protein beverages

EDF and ELF were added singly to a variety of beverage systems (water, AJ, LL, LL Free, IT, BT, and NAB) at four different concentrations, 0%, 2.5%, 5% and 10%. These concentrations were chosen to match the protein content of protein soft drinks that were available on the global market from a previous study performed by Ahern et al. (2023) (i.e. for commercial relevance). According to the protein content of the ingredients Ahern et al. (2025) each beverage had a final protein content of approximately 2%, 4% and 8% with the addition of 2.5%, 5% and 10% (w/w) of ingredient, respectively. Protein solubility was done on ingredients exceeding 80% across pH 2 – 9, relevant to soft drinks acidic value. A range of different drinks were chosen to cover a variety of beverage matrices. Carbonated beverages (LL, LL Free, NAB) were

decarbonated before protein addition using ultrasonication as described by Ahern et al. (2023). The BT was prepared using package instructions by brewing 2 teabags in 1 L of boiled water for 4 min, followed by the removal of the teabags and cooling until it reached room temperature (~20°C). The beverages were mixed using a vortex, then left for 2 hrs to allow further hydration, followed by a 30 s mixing stage using an Ultra-Turrax equipped with a S10N-10G dispersing element at speed 3 (IKA Labortechnik, Janke and Kunkel GmbH, Staufen, Germany). All beverages were freshly prepared prior to analysis.

5.2.3. pH

The pH of each beverage was measured using a calibrated pH meter (Mettler Toledo, Ohio, United States) at 20°C.

5.2.4. Apparent Viscosity

Rheological behaviour was determined using a rheometer (MCR301, Anton Paar GmbH, Graz, Austria) equipped with a concentric cylinder measuring attachment (Anton Paar GmbH, Graz, Austria) as described by Ahern et al. (2023). A shear sweep was performed, measured as a function of shear rate ranging from 0.5 to 100 (1/s) at 20°C. The apparent viscosity at 80.7 (1/s) was evaluated and used to compare the beverages.

5.2.5. Liquid Density

The liquid density (g/cm^3) of the soft drinks was determined using the Anton Paar density meter DMA 4500 M (Anton Paar GmbH, Austria). The sample was loaded and measured based on the oscillating U-shaped-tube principle where the sample is excited and oscillated at an oscillation period specific for the sample mass and volume at 20°C.

5.2.6. Turbidity

Turbidity was measured using a turbidity meter (Thermo Scientific Eutech TN-100 Waterproof Turbidimeter, Singapore, Singapore). Samples were measured at a wavelength of 850 nm at 20°C. A sample volume of 10 ml was used to determine turbidity ranging from 0 – 2000 nephelometric turbidity unit (NTU).

5.2.7. Dispersion Stability

Dispersion stability was determined using an analytical centrifuge (LUMiSizer, LUM GmbH, Berlin, Germany), through phase separation based on light transmission during centrifugation. The separation rate, an indication of stability was reported. The cycle configuration involved taking 100 profiles in 10 s intervals during centrifugation at 1000 rcf for 17 min, at a

wavelength of 865 nm and a temperature of 20°C. The percentage of integrated light transmission increased over time and the separation rate in is expressed as the % transmission per minute, as reported by Vogelsang-O'Dwyer et al. (2021).

5.2.8. Particle Size Distribution

The average particle size (nm) and polydispersity index (PDI) of each soft drink was measured using dynamic light scattering technology with the Zetasizer Nano Z (Malvern Instruments Ltd., Worcestershire, UK), equipped with a 633 nm laser and set size range of 0.3 nm – 10 µm. A refractive index of 1.45 and absorbance at 0.001 specific for proteins was used at 20°C.

5.2.9. Foam Capacity and Foam Stability

Foaming properties of the beverages were determined by frothing 20 ml of samples using an Ultra-Turrax equipped with a S10N-10G dispersing element (IKA Labortechnik, Janke and Kunkel GmbH, Staufen, Germany), at the maximum speed of 6 for 30 s. Sample expansion was calculated using the following equations (Vogelsang-O'Dwyer, et al., 2020):

$$Foaming\ capacity\ (\%) = \left(\frac{Foam\ height\ immediately\ after\ foaming}{Initial\ sample\ height}\right) \times 100$$

$$Foam\ stability\ (\%) = \left(\frac{Foam\ height\ after\ 1\ hour}{Foam\ height\ immediately\ after\ foaming}\right) \times 100$$

5.2.10. Tribology

Tribological measurements were conducted on a rheometer MCR301 using the BC-12.7 ball-on-3-pins tribology attachment (Anton Paar GmbH, Graz, Austria) at 37°C as described by Fox et al. (2022). The attachment contains changeable tribopairs (a glass ball and polydimethylsiloxane (PDMS) pins), which were supplied by Anton Paar (Graz, Austria). New pins were used for each test consisting of one run-in period and two repeated measurements of Stribeck curves. Before starting a test, the pin-holder and ball-holder were washed gently using a dilute detergent solution and rinsed thoroughly as well as being rinsed in acetone and dried with lab-wipes with the tribopairs. The test sequence was as followed: first the glass ball was lowered until a target force of 3 N was reached and held at that height for an equilibration period of 1 min. Furthermore, the following settings of the measurement system were selected: a logarithmic increase in sliding speed from 10^{-8} to 1 m/s was performed recording 80 data points with point duration from 1 to 10 s logarithmic, followed by a resting period of 1 min. Only the 0% and the 10% (w/w) beverages of EDF and ELF were measured using 1 ml aliquots. Three consecutive Stribeck curve measurements were performed for each sample. The first

measurement served as a run-in period and was excluded from the data set. Each sample was performed in triplicate, resulting in six evaluated datasets per sample.

5.2.11. Statistics

All analyses were performed in triplicate. Statistical analysis was performed using Origin lab pro2025b (Northampton, MA, USA). A one-way ANOVA with post hoc Tukey test ($p < 0.05$), principal component analysis, as well as a correlation analysis were performed.

5.3. Results

The results are separated into two parts. First, the changes in techno-functionality due to protein fortification with EDF and ELF at different concentrations are reported. The second part reveals the degree of changes of the beverages due to either EDF or ELF, illustrated in a principal component analysis (PCA) biplot.

5.3.1. Impact of protein fortification on techno-functionality of beverages

Table 5.2: Physicochemical properties of soft drinks including EverPro Dark Fraction (D) or EverPro Light Fraction (L). Statistical analysis was performed within the same drink identifying the impact of EverPro on the soft drinks' characteristics. Different lower-case letters within the same beverage type indicates significant differences ($p < 0.05$). n.a – non applicable.

	Soft Drink and EverPro inclusion level	pH	Turbidity (NTU)	Apparent Viscosity at 80.7 1/s (mPa.s)	Density (g/cm^3)	Particle size (nm)	Polydispersity index	Separation rate (%/min)	Foaming capacity (%)	Foaming stability (%)
Control	Water control (0%)	6.71 ± 0.00 ^{a}	0.16 ± 0.04 ^{a}	1.07 ± 0.13 ^{a}	0.9985 ± 0.0000 ^{a}	n.a	n.a	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
	Water D 2.5%	8.15 ± 0.02 ^{b}	25.7 ± 0.53 ^{b}	1.27 ± 0.13 ^{ab}	1.0061 ± 0.0001 ^{b}	298 ± 16 ^{d}	0.59 ± 0.04 ^{a}	0.09 ± 0.00 ^{b}	120.03 ± 3.53 ^{b}	36.9 ± 0.57 ^{f}
	Water D 5%	8.25 ± 0.05 ^{b}	42.73 ± 0.06 ^{c}	1.57 ± 0.08 ^{bc}	1.0138 ± 0.0000 ^{c}	306 ± 21 ^{cd}	0.65 ± 0.04 ^{a}	0.14 ± 0.00 ^{c}	128.99 ± 1.26 ^{bc}	37.07 ± 1.38 ^{f}
	Water D 10%	8.24 ± 0.05 ^{b}	58.53 ± 0.58 ^{d}	2.56 ± 0.17 ^{d}	1.0295 ± 0.0000 ^{d}	417 ± 7 ^{b}	0.57 ± 0.02 ^{a}	0.20 ± 0.00 ^{d}	121.92 ± 2.44 ^{b}	28.14 ± 1.94 ^{d}
	Water L 2.5%	7.45 ± 0.01 ^{c}	52.5 ± 0.17 ^{e}	1.36 ± 0.06 ^{ab}	1.0064 ± 0.0001 ^{e}	256 ± 9 ^{e}	0.64 ± 0.19 ^{a}	0.06 ± 0.00 ^{e}	134.12 ± 3.22 ^{c}	19.89 ± 0.19 ^{b}
	Water L 5%	7.45 ± 0.01 ^{c}	96.93 ± 0.78 ^{f}	1.81 ± 0.09 ^{c}	1.0143 ± 0.0001 ^{f}	338 ± 9 ^{c}	0.55 ± 0.05 ^{a}	0.11 ± 0.00 ^{f}	128.57 ± 1.10 ^{bc}	23.9 ± 1.33 ^{c}
	Water L 10%	7.45 ± 0.01 ^{c}	184.33 ± 0.58 ^{g}	3.29 ± 0.10 ^{e}	1.0304 ± 0.0001 ^{g}	562 ± 7 ^{a}	0.56 ± 0.08 ^{a}	0.17 ± 0.01 ^{g}	125.92 ± 7.91 ^{bc}	33.12 ± 0.65 ^{e}
Juice	Apple Juice control (0%)	3.51 ± 0.01 ^{a}	4.26 ± 0.05 ^{c}	1.23 ± 0.05 ^{f}	1.0448 ± 0.0000 ^{e}	2560 ± 48 ^{d}	1.00 ± 0.00 ^{a}	0.04 ± 0.00 ^{d}	26.64 ± 5.17 ^{c}	84.85 ± 5.25 ^{a}
	Apple Juice D 2.5%	4.26 ± 0.08 ^{b}	625 ± 22.07 ^{b}	1.89 ± 0.01 ^{e}	1.0517 ± 0.0001 ^{d}	4607 ± 1228 ^{c}	0.85 ± 0.26 ^{a}	5.26 ± 0.46 ^{b}	124.83 ± 2.50 ^{ab}	34.64 ± 1.17 ^{bc}
	Apple Juice D 5%	4.74 ± 0.02 ^{c}	360.67 ± 9.45 ^{d}	2.68 ± 0.47 ^{cd}	1.0586 ± 0.0001 ^{c}	2560 ± 491 ^{d}	0.22 ± 0.08 ^{b}	6.09 ± 0.19 ^{ab}	130.68 ± 2.54 ^{ab}	36.87 ± 1.70 ^{bc}
	Apple Juice D 10%	5.49 ± 0.06 ^{d}	466.67 ± 6.51 ^{e}	4.07 ± 0.25 ^{b}	1.0728 ± 0.0010 ^{b}	6509 ± 420 ^{bc}	0.83 ± 0.29 ^{a}	5.4 ± 0.13 ^{ab}	133.82 ± 2.37 ^{a}	43.99 ± 1.93 ^{b}
	Apple Juice L 2.5%	4.12 ± 0.01 ^{e}	670.33 ± 13.32 ^{b}	2.04 ± 0.06 ^{de}	1.052 ± 0.0001 ^{d}	8050 ± 1247 ^{b}	1.00 ± 0.00 ^{a}	5.82 ± 0.28 ^{ab}	120.23 ± 7.04 ^{b}	37.99 ± 6.19 ^{bc}
	Apple Juice L 5%	4.49 ± 0.01 ^{f}	548.67 ± 12.22 ^{f}	2.88 ± 0.28 ^{c}	1.0594 ± 0.0002 ^{c}	11180 ± 528 ^{a}	1.00 ± 0.00 ^{a}	6.18 ± 0.02 ^{a}	122.91 ± 3.64 ^{b}	32.01 ± 5.59 ^{cd}
	Apple Juice L 10%	4.98 ± 0.02 ^{g}	956.67 ± 41.31 ^{a}	5.43 ± 0.12 ^{a}	1.0743 ± 0.0001 ^{a}	834 ± 46 ^{d}	0.85 ± 0.13 ^{a}	1.21 ± 0.54 ^{c}	122.61 ± 4.90 ^{b}	21.22 ± 1.83 ^{d}
Soft drink (full sugar)	Lemon Lime control (0%)	3.30 ± 0.02 ^{f}	0.24 ± 0.11 ^{a}	1.25 ± 0.06 ^{d}	1.0175 ± 0.0000 ^{e}	917 ± 219 ^{b}	0.45 ± 0.13 ^{c}	0.02 ± 0.00 ^{c}	0.00 ± 0.00 ^{e}	0.00 ± 0.00 ^{f}
	Lemon Lime D 2.5%	5.03 ± 0.06 ^{d}	1768.67 ± 13.61 ^{b}	1.59 ± 0.05 ^{de}	1.0249 ± 0.0001 ^{d}	3354 ± 324 ^{a}	1.00 ± 0.00 ^{a}	2.84 ± 0.30 ^{a}	118.05 ± 3.77 ^{cd}	38.41 ± 1.49 ^{b}
	Lemon Lime D 5%	5.68 ± 0.02 ^{b}	617.67 ± 14.01 ^{d}	1.86 ± 0.17 ^{cd}	1.0325 ± 0.0001 ^{c}	943 ± 80 ^{b}	0.22 ± 0.03 ^{d}	0.49 ± 0.04 ^{b}	113.57 ± 1.16 ^{d}	35.22 ± 0.62 ^{bc}
	Lemon Lime D 10%	6.57 ± 0.02 ^{a}	770.33 ± 62.31 ^{c}	2.81 ± 0.27 ^{b}	1.0473 ± 0.0005 ^{b}	338 ± 14 ^{b}	0.72 ± 0.06 ^{b}	0.19 ± 0.02 ^{bc}	142.64 ± 2.34 ^{a}	45.36 ± 2.03 ^{a}
	Lemon Lime L 2.5%	4.65 ± 0.0 ^{e}	< 2000	1.80 ± 0.04 ^{d}	1.0249 ± 0.0001 ^{d}	3977 ± 1342 ^{a}	1.00 ± 0.00 ^{a}	2.68 ± 0.11 ^{a}	124.68 ± 2.96 ^{bc}	29.64 ± 1.49 ^{d}
	Lemon Lime L 5%	5.07 ± 0.01 ^{d}	807.00 ± 37.24 ^{c}	2.26 ± 0.03 ^{c}	1.0325 ± 0.0003 ^{c}	253 ± 11 ^{b}	0.50 ± 0.04 ^{c}	0.13 ± 0.00 ^{c}	124.44 ± 2.22 ^{b}	23.23 ± 2.05 ^{e}
	Lemon Lime L 10%	5.56 ± 0.01 ^{c}	165.00 ± 37.36 ^{e}	3.98 ± 0.27 ^{a}	1.0482 ± 0.0002 ^{a}	406 ± 20 ^{b}	0.67 ± 0.03 ^{b}	0.20 ± 0.01 ^{bc}	123.74 ± 2.06 ^{bc}	34.31 ± 0.36 ^{c}

Soft drink (no sugar)	Free Lemon Lime control (0%)	3.30 ± 0.02 ^{f}	0.38 ± 0.25 ^{a}	1.04 ± 0.03 ^{e}	0.9997 ± 0.0000 ^{e}	1035 ± 110 ^{cd}	0.47 ± 0.13 ^{c}	0.03 ± 0.00 ^{c}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{c}
	Free Lemon Lime D 2.5%	4.95 ± 0.02 ^{d}	927.00 ± 52.85 ^{b}	1.39 ± 0.03 ^{d}	1.0072 ± 0.0000 ^{d}	3981 ± 742 ^{a}	1.00 ± 0.00 ^{a}	3.31 ± 0.05 ^{a}	122.17 ± 3.55 ^{a}	36.83 ± 3.21 ^{a}
	Free Lemon Lime D 5%	5.63 ± 0.01 ^{b}	984.00 ± 70.00 ^{b}	1.72 ± 0.06 ^{c}	1.0149 ± 0.0001 ^{c}	1571 ± 38 ^{c}	0.80 ± 0.04 ^{ab}	1.82 ± 0.10 ^{b}	121.74 ± 2.17 ^{a}	36.31 ± 2.59 ^{a}
	Free Lemon Lime D 10%	6.48 ± 0.04 ^{a}	564.67 ± 39.80 ^{c}	2.54 ± 0.18 ^{b}	1.0303 ± 0.0009 ^{b}	319 ± 18 ^{d}	0.65 ± 0.07 ^{bc}	0.21 ± 0.01 ^{c}	127.25 ± 9.12 ^{a}	38.61 ± 3.13 ^{a}
	Free Lemon Lime L 2.5%	4.65 ± 0.00 ^{e}	< 2000	1.64 ± 0.08 ^{cd}	1.0073 ± 0.0001 ^{d}	2668 ± 671 ^{b}	1.00 ± 0.13 ^{a}	3.23 ± 0.14 ^{a}	122.46 ± 1.26 ^{a}	17.75 ± 1.79 ^{b}
	Free Lemon Lime L 5%	5.08 ± 0.01 ^{c}	267.33 ± 13.61 ^{d}	1.82 ± 0.12 ^{c}	1.0153 ± 0.0001 ^{c}	277.07 ± 27 ^{d}	0.54 ± 0.01 ^{c}	0.21 ± 0.02 ^{c}	120 ± 6.14 ^{a}	18.95 ± 1.27 ^{b}
	Free Lemon Lime L 10%	5.60 ± 0.01 ^{b}	189.67 ± 32.52 ^{d}	3.41 ± 0.05 ^{a}	1.0314 ± 0.0003 ^{a}	360 ± 46 ^{d}	0.67 ± 0.01 ^{bc}	0.19 ± 0.01 ^{c}	120.29 ± 6.28 ^{a}	35.04 ± 2.96 ^{a}
Sweetened tea-based beverage	Ice Tea control (0%)	3.10 ± 0.00 ^{a}	23.67 ± 0.42 ^{d}	1.09 ± 0.13 ^{f}	1.0177 ± 0.0000 ^{a}	187 ± 31 ^{c}	0.30 ± 0.07 ^{bc}	0.04 ± 0.01 ^{e}	24.45 ± 2.37 ^{e}	47.22 ± 4.81 ^{a}
	Ice Tea D 2.5%	4.71 ± 0.01 ^{b}	980.00 ± 3.61 ^{a}	1.54 ± 0.12 ^{e}	1.025 ± 0.0001 ^{b}	3543 ± 365 ^{a}	0.58 ± 0.43 ^{abc}	4.16 ± 0.27 ^{b}	117.3 ± 2.56 ^{d}	36.81 ± 1.89 ^{bc}
	Ice Tea D 5%	5.44 ± 0.02 ^{c}	953.33 ± 16.86 ^{a}	1.93 ± 0.14 ^{cd}	1.0326 ± 0.0001 ^{c}	3621 ± 540 ^{a}	0.94 ± 0.10 ^{a}	2.76 ± 0.23 ^{c}	118.3 ± 3.69 ^{cd}	30.25 ± 1.20 ^{cd}
	Ice Tea D 10%	6.68 ± 0.02 ^{d}	82.03 ± 2.80 ^{d}	3.09 ± 0.06 ^{b}	1.0479 ± 0.0000 ^{d}	366 ± 47^{c}	0.71 ± 0.01 ^{ab}	0.19 ± 0.01 ^{e}	122.46 ± 1.26 ^{bcd}	34.92 ± 2.25 ^{bcd}
	Ice Tea L 2.5%	4.53 ± 0.01^{e}	1006.33 ± 54.86 ^{a}	1.73 ± 0.10 ^{de}	1.0252 ± 0.0000 ^{e}	2427± 515 ^{b}	0.15 ± 0.05 ^{c}	5.16 ± 0.07 ^{a}	125.02 ± 1.63 ^{abc}	29.75 ± 3.05 ^{d}
	Ice Tea L 5%	4.99 ± 0.01 ^{f}	781.00 ± 12.12 ^{b}	2.25 ± 0.03 ^{c}	1.0328 ± 0.0001 ^{f}	1736± 182 ^{b}	0.89 ± 0.20 ^{a}	0.98 ± 0.10 ^{d}	128.6 ± 3.63 ^{ab}	19.44 ± 0.71 ^{e}
	Ice Tea L 10%	5.56 ± 0.03 ^{g}	186.00 ± 4.36 ^{c}	4.21 ± 0.19 ^{a}	1.0485 ± 0.0002 ^{g}	341 ± 4 ^{c}	0.54 ± 0.00 ^{abc}	0.20 ± 0.01 ^{e}	131.16 ± 1.26 ^{a}	39.23 ± 0.84 ^{b}
Tea (unsweetened)	Black Tea control (0%)	4.88 ± 0.02 ^{a}	27.47 ± 1.25 ^{f}	1.10 ± 0.06 ^{e}	0.9995 ± 0.0000 ^{a}	309 ± 28 ^{d}	0.68 ± 0.07 ^{a}	0.08 ± 0.01 ^{e}	27.95 ± 2.75 ^{d}	63.1 ± 4.29 ^{a}
	Black Tea D 2.5%	7.56 ± 0.01 ^{b}	789.00 ± 12.29 ^{c}	1.33 ± 0.06 ^{de}	1.0071 ± 0.0000 ^{b}	145 ± 7 ^{d}	0.14 ± 0.01 ^{b}	0.25 ± 0.02 ^{e}	129.63 ± 3.39 ^{ab}	40.55 ± 1.70 ^{b}
	Black Tea D 5%	7.72 ± 0.04 ^{c}	737.67 ± 12.06 ^{de}	1.57 ± 0.08 ^{cd}	1.0144 ± 0.0005 ^{d}	191 ± 5 ^{d}	0.15 ± 0.02 ^{b}	0.35 ± 0.01 ^{de}	131.2 ± 3.09 ^{a}	39.23 ± 0.84 ^{b}
	Black Tea D 10%	7.79 ± 0.02 ^{d}	699.67 ± 13.01 ^{e}	2.30 ± 0.04 ^{b}	1.0303 ± 0.0001 ^{e}	2337 ± 76 ^{a}	0.14 ± 0.01 ^{b}	0.90 ± 0.09 ^{cd}	121.25 ± 4.01 ^{bc}	35.69 ± 0.68 ^{bc}
	Black Tea L 2.5%	6.83 ± 0.02 ^{e}	1641.33 ± 13.8 ^{a}	1.36 ± 0.07 ^{de}	1.0073 ± 0.0001 ^{b}	197 ± 6 ^{d}	0.15 ± 0.01 ^{b}	1.31 ± 0.00 ^{c}	130.94 ± 1.48 ^{a}	18.14 ± 1.81 ^{d}
	Black Tea L 5%	7.20 ± 0.01 ^{f}	765.67 ± 17.62 ^{cd}	1.80 ± 0.14 ^{c}	1.0152 ± 0.0000 ^{c}	607 ± 120 ^{c}	0.26 ± 0.02 ^{b}	5.13 ± 0.29 ^{a}	122.65 ± 3.54 ^{abc}	30.93 ± 2.08 ^{c}
	Black Tea L 10%	7.32 ± 0.03 ^{g}	1151.33 ± 20.50 ^{b}	3.42 ± 0.27 ^{a}	1.0309 ± 0.0003 ^{f}	1809 ± 215 ^{b}	0.51 ± 0.19 ^{a}	3.53 ± 0.43 ^{b}	118.72 ± 2.62 ^{c}	30.89 ± 1.26 ^{c}
Malt-based beverage	Non Alcoholic Beer control (0%)	4.32 ± 0.00 ^{a}	5.05 ± 0.15 ^{e}	1.46 ± 0.05 ^{c}	1.0156 ± 0.0000 ^{d}	465 ± 47 ^{c}	0.55 ± 0.03 ^{ab}	0.03 ± 0.00 ^{e}	121.75 ± 0.47 ^{bc}	39.91 ± 3.27 ^{a}
	Non Alcoholic Beer D 2.5%	6.01 ± 0.02 ^{b}	766.33 ± 9.29 ^{a}	1.77 ± 0.06 ^{c}	1.0227 ± 0.0002 ^{c}	2472 ± 305 ^{a}	0.16 ± 0.09 ^{c}	1.03 ± 0.02 ^{a}	124.09 ± 2.20 ^{bc}	35.32 ± 2.39 ^{a}
	Non Alcoholic Beer D 5%	6.64 ± 0.03 ^{c}	258.33 ± 23.16 ^{b}	2.06 ± 0.18 ^{c}	1.0306 ± 0.0004 ^{b}	380 ± 40 ^{c}	0.57 ± 0.04 ^{ab}	0.15 ± 0.01 ^{c}	117.39 ± 2.17 ^{c}	26.59 ± 4.31 ^{b}
	Non Alcoholic Beer D 10%	7.27 ± 0.01 ^{d}	112.10 ± 33.05 ^{d}	3.19 ± 0.32 ^{b}	1.0457 ± 0.0006 ^{a}	614 ± 88 ^{bc}	0.51 ± 0.12 ^{b}	0.19 ± 0.01 ^{b}	133.36 ± 3.58 ^{a}	41.3 ± 1.31 ^{a}
	Non Alcoholic Beer L 2.5%	5.26 ± 0.01 ^{e}	88.90 ± 1.73 ^{d}	1.89 ± 0.06 ^{c}	1.0231 ± 0.0001 ^{c}	336 ± 27 ^{c}	0.71 ± 0.05 ^{a}	0.12 ± 0.0 ^{d}	126.35 ± 4.39 ^{ab}	11.55 ± 0.89 ^{c}
	Non Alcoholic Beer L 5%	5.62 ± 0.02 ^{f}	92.97 ± 1.96 ^{d}	2.30 ± 0.14 ^{c}	1.0309 ± 0.0000 ^{b}	355 ± 56 ^{c}	0.6 ± 0.12 ^{ab}	0.16 ± 0.01 ^{c}	118.15 ± 2.83 ^{c}	5.52 ± 0.06 ^{c}
	Non Alcoholic Beer L 10%	6.08 ± 0.01 ^{g}	163.33 ± 1.53 ^{c}	4.82 ± 0.33 ^{a}	1.0464 ± 0.0003 ^{a}	973 ± 177 ^{b}	0.44 ± 0.02 ^{b}	0.21 ± 0.00 ^{b}	125.89 ± 1.88 ^{ab}	25.11 ± 2.04 ^{b}

5.3.1.1. pH

The pH values of the beverages are displayed in **Table 5.2**. All beverages were quite different in their pH values, with the lowest pH values observed for IT (3.10) followed by LL (3.30), LL Free (3.30) and AJ (3.51). The pH of NAB (4.32) and BT (4.88) are slightly higher, while the highest pH value was observed for water (6.71).

The addition of EDF and ELF caused an increase in pH, with ELF elevating the value to a higher extend compared to ELF. Increased inclusion of either EP™ ingredient consistently elevated the pH of all beverages significantly. However, in water, the addition of 2.5% of either EDF or ELF caused an increase in pH from 6.71 (0% addition) to 8.15 and 7.45, respectively, but higher addition levels did not further raise the pH. The highest pH values (>7) in beverages fortified with 10% of EDF were observed in water (8.25) followed by BT (7.8) and NAB (7.3). The addition of 10% EDF to acidic beverages elevated their pH to 6.7 in IT, to 6.6 in LL, to 6.5 in LL Free, and to 5.5 in AJ. Addition of ELF followed the same trend in these beverages with slightly lower final pH values. A significant strong positive correlation between the addition level of either EP ingredient in IT and the resulting pH was observed ($r = 0.97$, $p \leq 0.05$). Interestingly, the addition of ELF in AJ showed a strong correlation between addition level and pH ($r = 0.97$, $p \leq 0.05$), but fortifying with EDF did not show significance.

5.3.1.2. Apparent Viscosity

The formulation of a beverage can impact viscosity, which is an important functional property capable of influencing the mouthfeel/texture. The apparent viscosity of the beverages at 80.7 1/s are presented in **Table 5.2**. Among the controls, LL Free exhibited the lowest viscosity (1.04 mPa·s) followed by water (1.07 mPa·s), IT (1.09 mPa·s), and BT (1.10 mPa·s). The highest viscosity among the controls were found in NAB (1.46 mPa·s) followed by LL (1.25 mPa·s) and AJ (1.23 mPa·s).

Fortification with both EDF and ELF increased apparent viscosity across all beverages, with ELF addition resulting in higher viscosity values than EDF. Water showed a clear increase in viscosity with EP addition, reaching significantly higher values of 3.19 mPa·s (EDF) and 4.82 mPa·s (ELF) at 10% inclusion. AJ with 10% inclusion was shown to be the most viscous beverage, particularly with ELF (5.43 mPa·s), followed by EDF (4.07 mPa·s) with statistical significance. LL showed moderate increases, with 10% addition of EDF and ELF resulting in viscosity values of 2.81 mPa·s and 3.98 mPa·s, respectively**.** LL Free increased to 2.54 mPa·s

(EDF) and 3.41 mPa·s (ELF) at 10% inclusion levels. NAB displayed apparent viscosities of 3.19 mPa·s (EDF) and 4.82 mPa·s (ELF) at 10% ingredient addition, with ELF inclusion resulting in a significantly higher value than EDF. Additionally, the inclusion of either EP ingredient showed a higher impact on IT than on BT.

All beverages demonstrated a significantly strong positive correlation (r = 0.96 – 1.00, $p \leq$ 0.05) between protein inclusion level and apparent viscosity, with the exception of EDF addition in IT where the correlation was not significant.

5.3.1.3. Liquid Density

Density is an important physical parameter reflecting the content of soluble solids of a soft drink, meaning the density of a solution is dependent on the concentration of dissolved substances within the product. The impact of EDF and ELF fortification on beverage density is illustrated in **Table 5.2**. As a reference value, water showed an average density value of 1 g/cm^3. Regarding the controls, beverages containing sugars exhibited higher densities compared to sugar free beverages; AJ (1.0448 g/cm^3), LL (1.0175 g/cm^3), IT (1.0177 g/cm^3) and NAB (1.0156 g/cm^3), whereas LL Free (0.9997 g/cm^3) and BT (0.9995 g/cm^3) displayed lower densities.

Fortification with both EP ingredients led to elevated density values in all beverages, while ELF showed significantly higher density values compared to EDF. Water with 10% addition of EP ingredients resulted in the lowest density values at 1.0295 g/cm^3 and 1.0304 g/cm^3, similar to BT at 1.0303 g/cm^3 and 1.0309 g/cm^3 for EDF and ELF inclusion, respectively. LL and LL Free reached similar densities at 10% inclusion with both EPs (1.05 g/cm^3 (EDF) and 1.03 g/cm^3(ELF)). NAB and IT increased to 1.046 g/cm^3 and 1.048 g/cm^3 at 10% EP inclusion. AJ showed the highest densities at 10% addition level, with a value of 1.0728 g/cm³ determined for EDF addition, and 1.0743 g/cm³ for ELF. addition

All beverages displayed a strong positive correlation between protein inclusion and density (r = 0.99, $p \leq 0.05$), revealing higher amounts of soluble solids with increasing EP fortification.

5.3.1.4. Turbidity

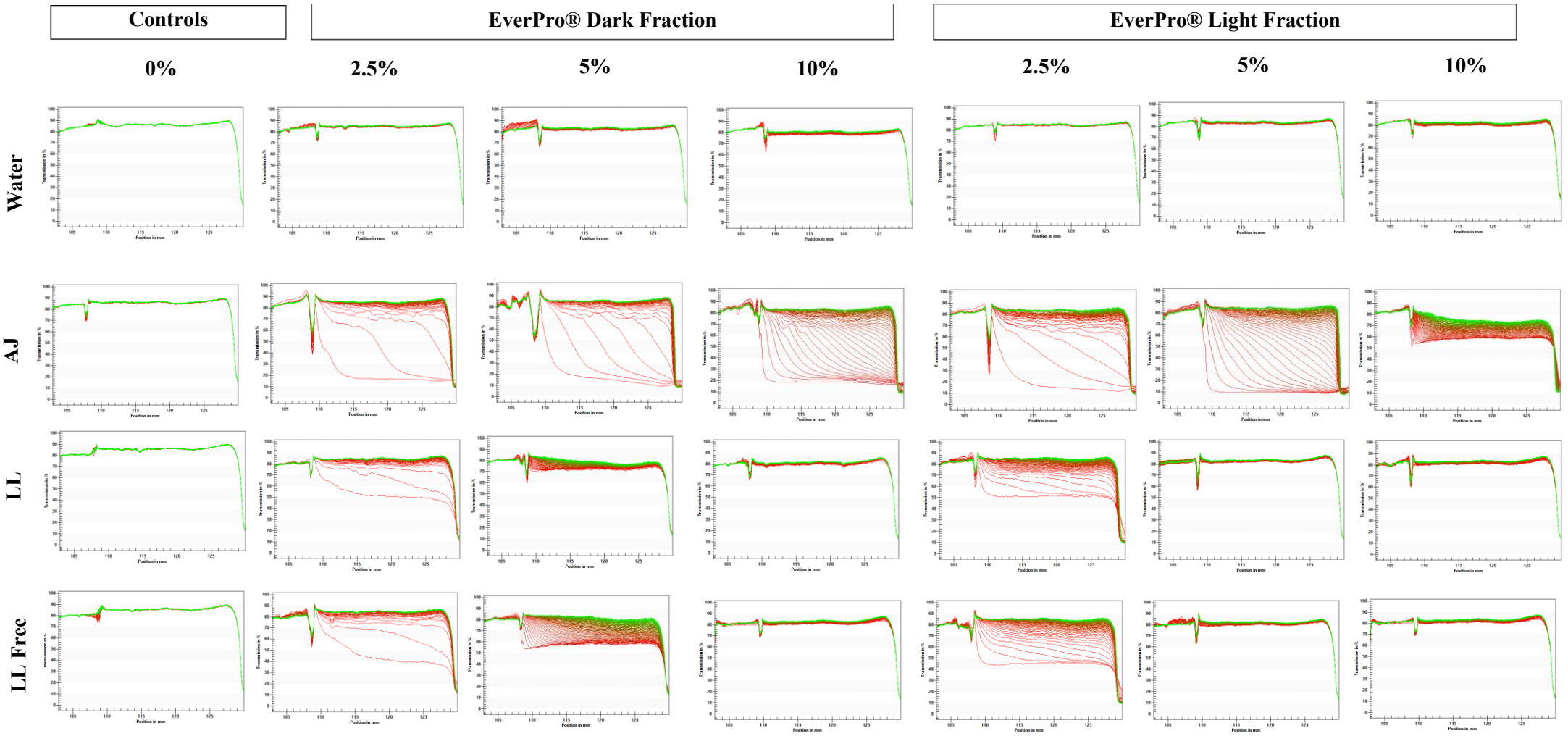
Turbidity reflects the clarity and transparency of a beverage. The turbidity results are displayed in **Table 5.2**. Control samples (0% inclusion) exhibited low turbidity, ranging from 0.2 – 27.5 NTU, with water being the lowest and BT the highest.

Addition of both EP ingredients significantly increased turbidity in all beverages. Water samples with EP inclusion consistently exhibited low turbidity across all concentrations, with ELF causing slightly higher values than EDF. The turbidity of most beverages was dependent on concentration levels. The most substantial increases in turbidity were observed in the LL beverages fortified with ELF at 2.5%, giving values out of range (≥ 2000 NTU). An addition of 2.5% EDF to LL showed higher turbidity (1768 ± 14 NTU) compared to LL Free (927 ± 53 NTU). IT and BT also showed high turbidity levels, especially at 2.5% inclusion of both EP ingredients. However, ELF caused higher turbidity in IT and BT than EDF. In AJ, the fortification with EDF caused the highest turbidity value at an inclusion level of 2.5%, while fortification with ELF reached the highest turbidity at 10% addition level. The fortification of NAB with EP ingredients displayed moderate turbidity increases. The addition of ELF to NAB revealed a significantly strong positive correlation with turbidity ($r = 0.95$, $p \leq 0.05$). Overall lower protein inclusions (2.5%) generally resulted in the most turbid samples (excluding water), while the higher inclusion level of 10% often reduced turbidity, observed in LL (EDF and ELF), LL Free (EDF and ELF), IT (EDF and ELF), BT (EDF) and NAB (EDF).

5.3.1.5. Dispersion Stability

An indication of dispersion stability is a low separation rate. The separation rate of each beverage is presented in **Table 5.2**. According to the light transmission profile (shown in **Figure 5.1**), all beverage controls (0% EP inclusion) showed low separation rates (≤ 0.08 %/min).

Water samples containing either EDF or ELF at all inclusion levels also showed similarly low values (≤ 0.2 %/min). Both EDF and ELF addition increased separation rates across all beverages with no consistent difference between the two ingredients. However, inclusion level showed a strong influence on separation behaviour. AJ showed sedimentation and a high separation rate at every EP concentration (highest in AJ ELF 5% = 6.18 ± 0.02 %/min). Both LL and LL Free followed a pattern similar to IT, where separation rates were the highest at low inclusion levels (2.5%), and significantly lower at 10%, indicating improved stability at higher protein levels. Dispersion stability was also influenced by 2.5% inclusion of both EPs in NAB. BT demonstrated a clear protein concentration-dependent effect for EDF, with a significant positive correlation observed between addition level and separation rate ($r = 0.98$, $p \leq 0.05$).


Controls
EverPro® Dark Fraction
EverPro® Light Fraction
0%
2.5%
5%
10%
2.5%
5%
10%
Water
AJ
LL
LL Free

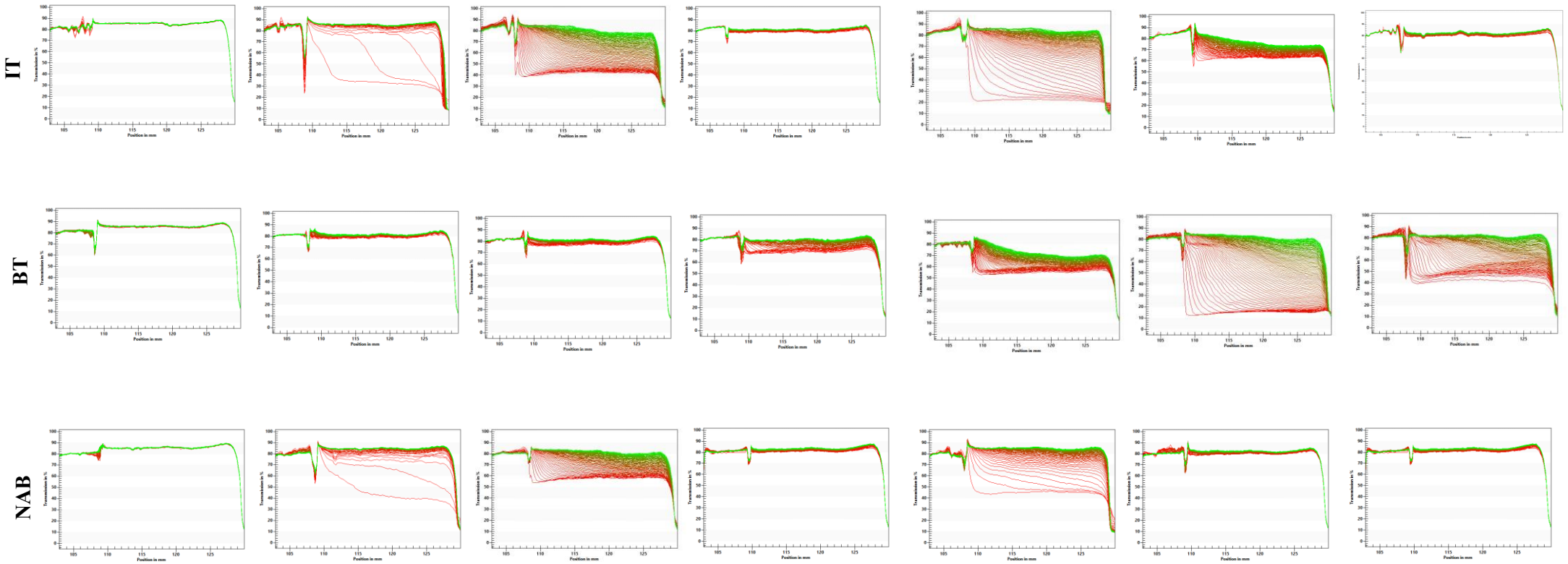


Figure 5.1: Lumisizer light transmission profiles of soft drinks containing 0%, 2.5%, 5%, 10% of EverPro Dark Fraction (EDF) or EverPro Light Fraction (ELF) as a function of time. (AJ) apple juice, (LL) lemon and lime, (LL Free) lemon and lime sugar free, (IT) ice tea, (BT) black tea, (NAB) non-alcoholic beer.

Across all beverages (excluding water), more than half of the samples displayed their highest separation rates at 2.5% protein inclusion, whereas the lowest rates were consistently observed at 10% addition, indicating greater stability at higher protein levels.

5.3.1.6. Particle Size Distribution

The average particle size (nm) and polydispersity index (PDI) values are presented in **Table 5.2**. Among the control beverages, water showed no detectable particle size. AJ exhibited the largest particle size (2560 ± 48.29 nm), followed by LL Free (1035 ± 110.4 nm) and LL (917 ± 218.67 nm). IT showed the smallest particle size (187 ± 30.64 nm), and BT (309 ± 27.64 nm) and NAB (465 ± 47.33 nm) also displayed comparatively low values. Across beverages, the addition of EP ingredients increased particle size, with no consistent differences observed between EDF or ELF. Increasing EP concentration did not lead to a uniform trend across the beverages. Water and BT were the only samples where increasing EP addition levels produced a clear trend. AJ with 2.5% and 5% fortification with ELF resulted in the highest values observed (8050 ± 1247 nm and 11180 ± 529 nm, respectively). LL soft drinks displayed a peak in particle sizes at 2.5% with both EPs (2700 – 4000 nm), followed by a marked decrease at 10% inclusion level (320 – 360 nm). IT showed a similar pattern with significant differences. NAB showed no clear relationship between inclusion level and particle size, with values varying independently of EP concentration.

Regarding PDI, the majority of the samples obtained a PDI > 0.5 (90% of all samples) indicating polydisperse systems with a wide particle size distribution.

5.3.1.7. Foam Capacity and Foam Stability

The foam capacity and stability were analysed, and the results are displayed in **Table 5.2**. Among the control samples, water, LL, and LL Free produced no measurable foam, while AJ, IT, and BT exhibited low foam capacities (26.64% ± 5.17, 24.45% ± 2.37, and 27.95% ± 2.75, respectively). NAB displayed the highest foaming capacity (121.75% ± 0.47) among the controls. Foam stability at 60 min was generally low, with values of 84.85% ± 5.25 (AJ), 47.22% ± 4.81 (IT), 63.10% ± 4.29 (BT), and 39.91% ± 3.27 (NAB) determined for the control beverages.

Inclusion of either EDF or ELF significantly increased the foam capacity and stability in all beverages compared to controls, except for NAB, which showed minimal changes. No consistent differences were observed between EDF and ELF across the beverages. Foam capacity ranged from 113.57% to 142.64% with protein inclusion, showing no clear correlation

with EP addition level in most beverages. Overall, lower and higher protein concentrations led to a similar foam capacity, indicating that foam formation was largely dependent on the presence of EP protein rather than the specific addition level. Despite the increases in foam capacity with EP fortification, foam stability remained below 50% in most samples.

5.3.1.8. Tribology

Soft tribology is a technique used to measure the frictional properties of food and beverages which can be linked with the friction between tongue and pallet leading to mouthfeel. **Figure 5.2** displays the Stribeck curves of beverages with EDF and ELF at 10% addition level. The presented curves for the beverages can be divided into static ($< 10^{-8} – 10^{-5}$ m/s), boundary ($10^{-5} – 10^{-4}$ m/s) and beginning of mixed ($> 10^{-4}$ m/s) regimes.

Water had the highest frictional factor compared to all other beverages, followed by the remaining beverage controls including LL Free, LL, NAB, AJ, IT and BT, showing frictional factors between 10^{-5} to 10^{-2}. although NAB and AJ showed higher frictional factors between sliding speeds 10^{-5} and 10^{-4}.

The inclusion of EP ingredients increased lubricating properties. ELF showed higher lubricating properties in the beverages compared to EDF. AJ exhibited the highest frictional properties when either of both ingredients were included. LL soft drinks showed similar Stribeck curves across static and boundary regimes. The addition of ELF, followed by EDF, to BT gave the highest lubricating properties. NAB showed the second highest lubricating properties followed by IT with EP inclusion.

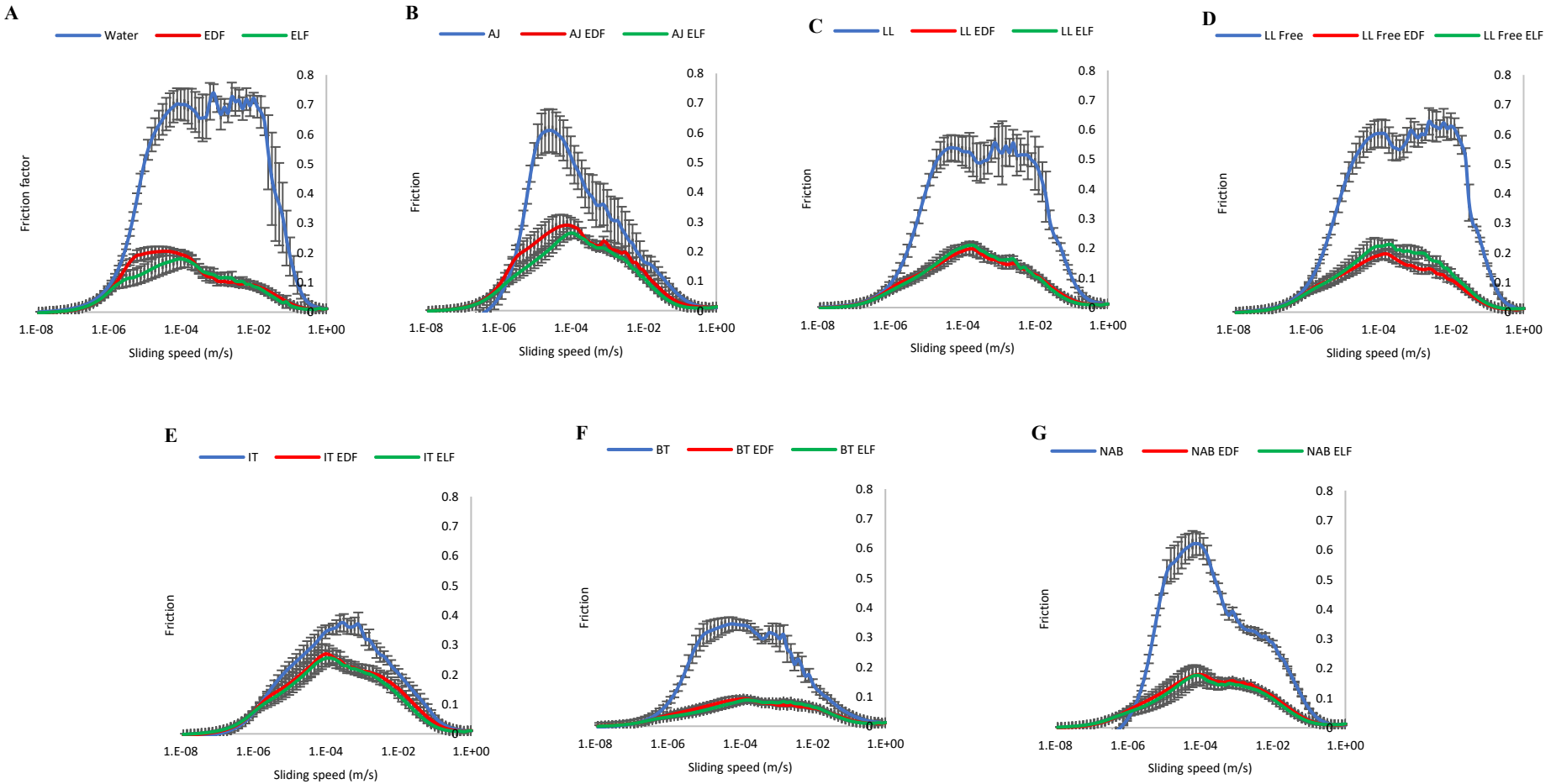
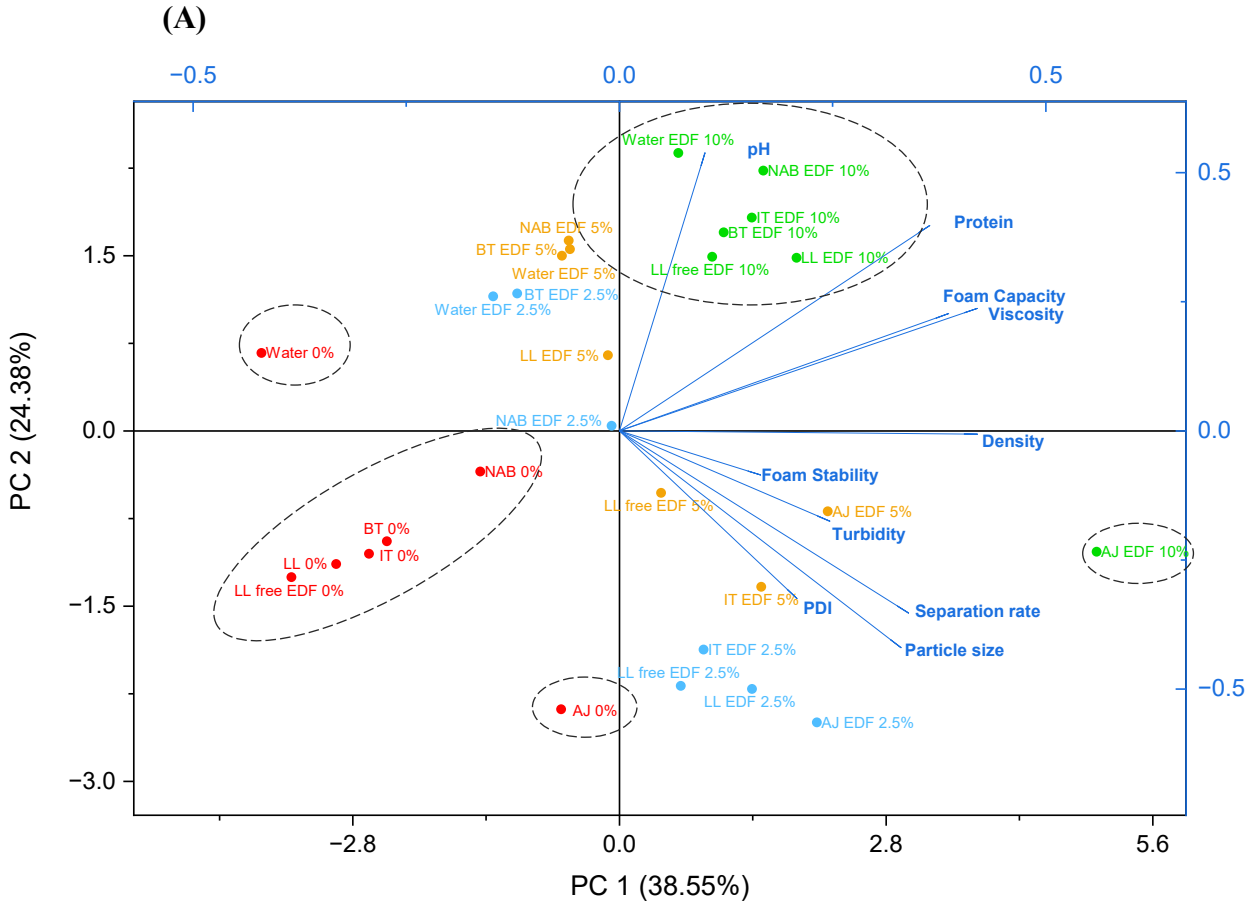


Figure 5.2: Stribeck curves of soft drinks containing EDF and ELF at 10%. Error bars indicate standard deviation. (A) water (Ahern et al., 2025), (B) apple juice, (C) lemon and lime, (D) lemon and lime sugar free, (E) ice tea, (F) black tea, (G) non-alcoholic beer.

5.3.1.9. Principal Component Analysis biplot

Two principal component analysis (PCA) biplots were performed to visualise the changes in beverage characteristics following fortification EDF (**Figure 5.3A**) or ELF (**Figure 5.3B**). The biplots captured a total variance of 62.93% for EDF fortification and 58.27% for ELF fortification.



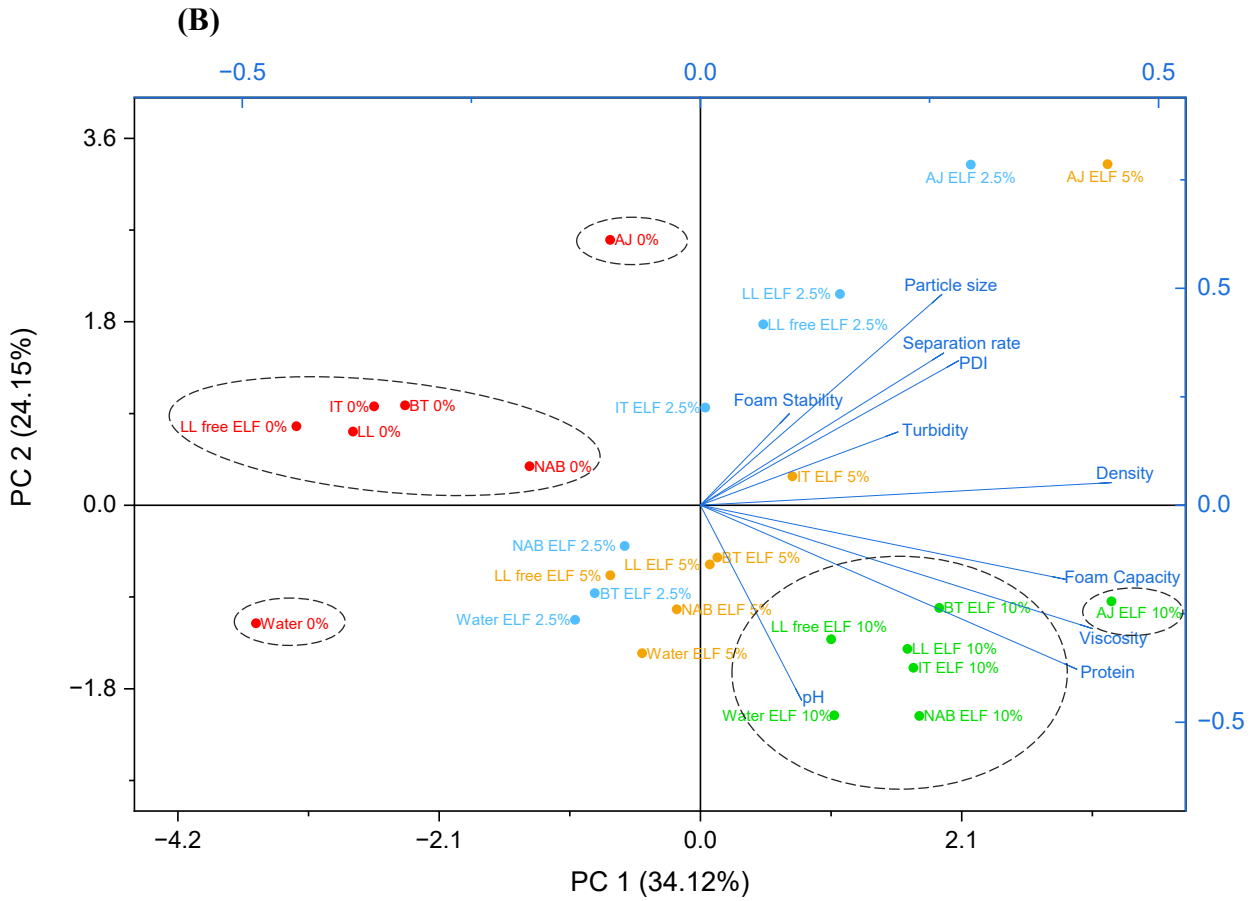


Figure 5.3: Principal component analysis biplot. (A) EverPro Dark (EDF) and (B) Light Fraction (ELF) at 0% (●), 2.5% (●), 5% (●) and 10% (●) ingredient inclusion level of beverages analysed including water, apple juice (AJ), lemon and lime (LL), lemon and lime sugar free (LL free), ice tea (IT) black tea (BT) and non-alcoholic beer (NAB).

The biplot illustrating the influence of EDF (**Figure 5.3A**) revealed five distinct groups including group 1: AJ 0%; group 2: water 0%; group 3: AJ EDF 10%; group 4: the remaining control (0%) beverages; group 5: the remaining 10% EDF beverages. Lower inclusion levels (2.5 and 5%) show the gradual modification of beverage techno functionality, with majority of samples clustering in the region associated with high dispersion instability properties. As EDF increased, a clear progression in sample distribution was observed. The distinct separation of AJ (0%) and water (0%) from the other beverages suggests notable differences in their techno-functional profiles. AJ with 10% EDF stands out distinctly due to its high density, separation rate, particle size, PDI and viscosity, of all which are positively correlating. The remaining 10% EDF beverages cluster together, sharing similar measurements for pH, foam capacity and turbidity, also positively correlating.

Similarly, the biplot in **Figure 5.3B** (influence of ELF) also showed five groups. Group 1: AJ 0%; group 2: water 0%; group 3: AJ ELF 10%; group 4: the remaining control (0%) beverages; and group 5: the remaining 10% ELF beverages. Increasing ELF concentration produced clear directional shifts in sample clustering, with higher inclusion levels (5% and 10%) associated with lower dispersion instability properties.

In both biplots, controls (indicated as "0%") clustered distinctly apart from samples with 10% EP ingredient addition, highlighting the impact of inclusion level on techno-functionality of the beverages. Hence, ingredients both show individual changes in beverage characteristics.

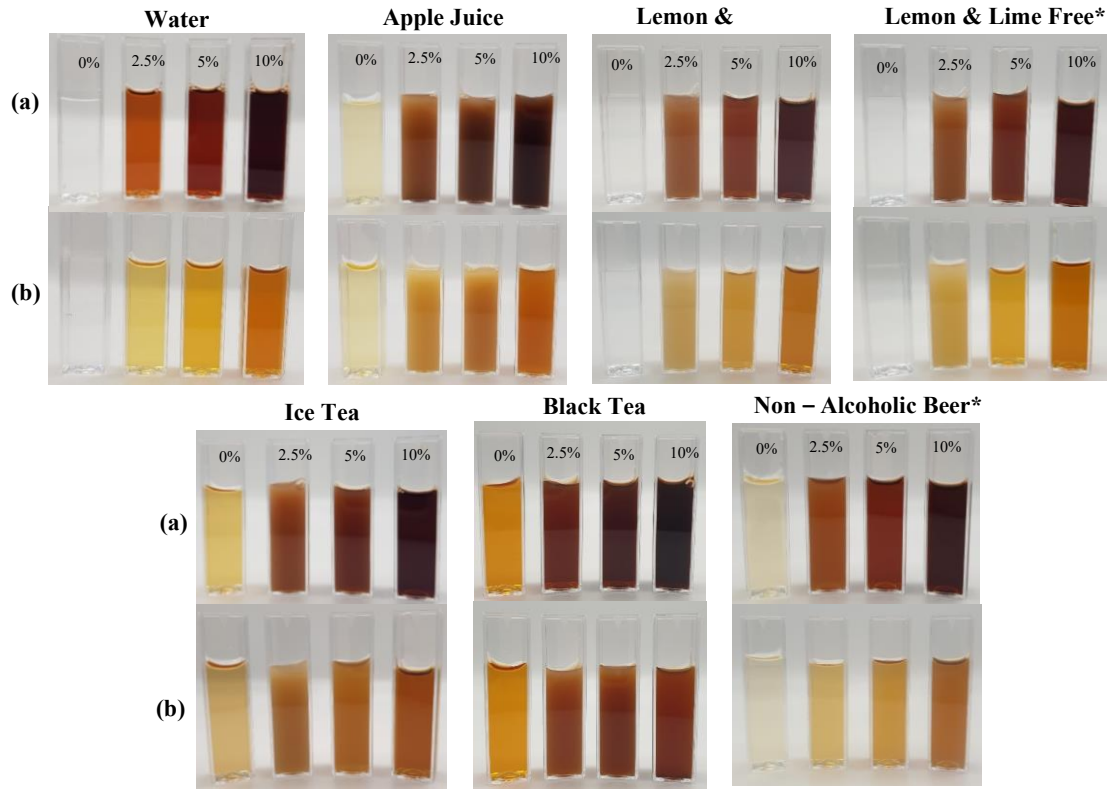


Figure 5.4: Images of beverages at all inclusion levels where row (a) EverPro Dark Fraction (EDF) and row (b) EverPro Light Fraction (ELF). An asterisk (*) indicates beverages that were decarbonated before analysis.

5.4. Discussion

The protein beverage market continues to expand, fuelled by health-conscious consumers seeking clear beverage alternatives that satisfy both hydration and nutritional requirements without the heaviness of protein smoothies or shakes. This trend highlights importance of developing protein ingredients that perform well in a variety of beverage systems. Numerous studies have explored fortifying fruit juices with protein to enhance nutritional value using primarily whey protein ingredients (i.e. concentrates, isolates and hydrolysates) at levels between ~ 0.8 to 6%, as well as collagen hydrolysate at a protein content of ~2.5% (Bilek & Bayram, 2015; Djurić et al., 2004; Goudarzi et al., 2015; Koffi et al., 2005; Prado et al., 2015; Yadav et al., 2016). The nutritional quality of EDF and ELF have been previously characterised with both ingredients containing a high protein content (>80%). All essential amino acids meet the FAO requirements for individuals above >3 years old with the exception of lysine, with notable levels of branch chain amino acids. Dynamic *in vitro* digestibility of EDF also demonstrated favourable amino acid digestibility with a DIAAS of 67%, exceeding values of other plant-based proteins studied (Ahern et al., 2025; Jaeger et al., 2024) Ahern et al. (2025) further demonstrated that these ingredients possess techno-functional properties that make them promising candidates for beverage applications. However, those findings were limited to testing in water. The present study addresses this gap by investigating both EP ingredients at various inclusion levels (2.5%, 5% and 10%) across a range of commonly consumed beverages, determining whether certain beverage properties pose challenges for incorporation. As these ingredients are upcycled from low cost BSG, they offer sustainable and economic advantages over proteins from dedicated crops like pea, faba or soy. However, ELF's extra processing increases its cost relative to EDF. Higher inclusion levels (2.5 – 10% w/w; 2-8g protein/100ml) cover cost effective moderate fortification to premium formulations consistent with market data showing higher protein contents and carbonated types were more expensive (Ahern et al., 2023). Pictures of each beverage are displayed in **Figure 5.4,** where clear differences can be seen between the inclusion of EDF and ELF. Fortifying beverages with EDF resulted in solutions with brown shade colours, increasing in darkness with increased addition level. The addition of ELF, on the other hand, caused a yellow shade colour at low concentrations, turning to dark orange and even brown colours at higher inclusion levels. This is, likely, due to the lighter colour of ELF powder, where the decolourisation process likely removed many of these Maillard reaction compounds (e.g. melanoidin) originating from the brewing process (Ahern

et al. (2025). However, residual pigments and their concentration showed to proportionally darken the beverages.

The majority of soft drinks have low pH values due to the addition of acidulants and, in the case of carbonated beverages, the presence of dissolved carbonic acid (Ashurst et al., 2017). EverPro® ingredients possess the unique functional property of being highly soluble over a range of pH values. At pH 4, the pH closest to their isoelectric points, both EDF and ELF exhibited high protein solubility values of 82% and 80% respectively (Ahern et al., 2025). The pH values of the chosen beverages (excluding water) fell within the acidic range of 3.1 – 4.9, as expected due to the presence of acidulants such as malic and citric acid (Ashurst et al., 2017). The beverages ranked by acidity from highest to lowest pH were as follows: IT > LL > LL Free > AJ > NAB > BT. The BT and NAB showed the lowest acidity amongst the beverages (excluding water). BT includes organic acids and polyphenols, such as tannins, developed from the oxidation/fermentation of tea leaves which can possess weakly acidic properties (Zhang et al., 2022). Additionally, NAB contains low amounts of organic acids synthesised during the brewing process (Fox et al., 2022; Nyhan et al., 2023). IT exhibited the highest acidity, attributed to a combination of organic acids, polyphenols from the black tea extract, and added acidulants (Ashurst et al., 2017; Zhang et al., 2022). Increased inclusion of both EP ingredients substantially increased the pH value closer to the neutral range. When both EP ingredients were gradually added to water, there was no change in average pH for all inclusion levels of EDF and ELF. Since proteins can act as a buffer, the addition of EP ingredients to a soft drink can interact with its acidic components. Specifically acidic amino acids with negatively charged side chains, such as aspartic and glutamic acid (~30g/100g of EP protein), can bind to the hydrogen ions in an acidic environment (Al-Dabbas et al., 2010). This interaction can reduce free hydrogen concentration, leading to an increase in pH within the beverage demonstrating their buffering capacity. Organic acids and ash can also contribute to a higher buffering capacity, where the slightly higher ash content observed in EDF compared to ELF may have influenced its elevated pH (Ahern et al., 2025; Al-Dabbas et al., 2010).

The liquid density of a beverage can be influenced by the amount of total soluble solids present (Jabeen et al., 2022). Additionally, soluble solids in a beverage system can elevate viscosity. Both density and viscosity showed similar trends. Beverage controls containing higher sugar contents (LL, AJ) exhibited higher density and viscosity results compared to those that used artificial sweeteners (IT, LL Free), as reported previously (Al-Dabbas et al., 2012; Saniah et al., 2012). When sugar is replaced with artificial sweeteners, the reduction in bulk or density

of the beverages leads to a significant decrease in viscosity, whereas if added at equivalent concentrations, these parameters remain comparable (Mittal & Bajwa, 2012). As artificial sweeteners can be 200 to 500 times sweeter than sucrose, only very minimal quantities are required (Ashurst, 2016) evident in the reduction of sugar from 4.7g in LL to 0g in LL Free, replaced by with aspartame and acesulfame K. Although the NAB is low in sugar, it showed the highest apparent viscosity, which is likely due to the presence of complex carbohydrates, such as beta glucan and dextrin from the malted barley, which contribute to viscosity (Fox et al., 2022). Higher concentrations of both EP ingredients resulted in increased density and viscosity values with significant positive correlations (with the exception of IT EDF for viscosity), as seen previously in fruit flavoured beverages fortified with whey protein ingredients (Yadav et al., 2016). Specifically, the addition of ELF led to higher density and viscosity values compared to EDF. The PCA biplots clearly demonstrates this by the distinct separate clusters of 0% and 10% inclusions. ELF went through further processing compared to EDF, particularly a decolourisation step. Decolourisation has been shown to alter the functional properties of proteins, particularly increasing water holding capacity, thereby increasing the viscosity (Yao et al., 2019). This occurs due to an oxidation process expanding the protein structure and allowing for the more efficient binding of water (Yao et al., 2019). Similar results have been observed for decolorised soy protein isolate, which also demonstrated increased water holding capacity (Sakai et al., 2022). EP originates from brewers spent grain and has been shown to consist of low molecular weight proteins and peptides (Ahern et al., 2025). This lack of large and folded structure of intact proteins can cause the lower water holding capacity leading to a lower change in viscosity. Additionally, ELF resulted in larger particle sizes in the water solution compared to EDF, which could be attributed to the different native pH values of the ingredients in water affecting protein charge. EDF has a higher pH than ELF, thus being further away from the isoelectric point (pI ~3.4), leading to higher repulsion forces and improved dispersion and/or hydration (Ahern et al., 2025). This was shown by Dissanayake et al. (2013) where whey protein dispersions exhibited increased viscosity with decreasing pH values, due to increased repulsive forces at higher pHs and enhanced aggregation of protein at lower pHs.

Stability in soft drinks is essential for maintaining consistent quality, appearance, and flavour throughout the products shelf life, ensuring consumer satisfaction. Many ready-to-drink protein beverages with both animal- and plant-based protein sources on the market tend to have neutral pHs with few being acidic (Ahern et al., 2023; Liu et al., 2021). Stabilizing plant-based protein

ingredients in an acidic environment can be a challenge as these proteins tend to precipitate around their isoelectric point, typically within this pH range. The pH of these beverages is important as it has been found that pH values far from their isoelectric point play a key role in the clearness of beverages because of the electrostatically repulsive forces amongst unfolded proteins or aggregated proteins/peptides (Laclair & Etzel, 2010). This was seen in the current study where EP inclusion level at 2.5% in soft drinks resulted in the most acidic pH values, and exhibited the highest turbidity and separation rate. On the other hand, 10% addition resulted in the highest pH values, with the clearest appearance and the lowest separation rates with little to no sedimentation. Particle size trends indicated that the largest size was observed at the 2.5% addition, with significant correlations not observed with increased inclusion levels. As seen in the PCA biplots (**Figure 5.3**), AJ is distinctly separated at both 0% and 10% inclusions due to its high particle size and separation rate, likely attributed to its natural composition including residual pulp which increases particle size, thus affecting stability as highlighted by Zhu et al. (2020). A study by Goudarzi et al. (2015) demonstrated that adjusting the pH to an optimum value resulted in a reduction in the mean particle size for both whey protein isolate/hydrolysate, with beverages with smaller particle sizes showing the clearest appearance. One method of trying to stabilize an acidic protein beverage can be the use of different polysaccharides such as beta glucan, low methoxyl pectin and chitosan that can alter the charge characteristics, potentially increasing stability (Zhang et al., 2023). The presence of beta glucan in the NAB beverages may explain the low separation rates observed across the varying EP concentrations. Another study conducted by Liu et al. (2020) investigated the use of six different hydrocolloids (as stabilizing agents) to prevent phase separation, where particle size decreased with increasing concentrations of some stabilizers.

While raising the pH can improve solubility and stability of proteins, it may also impact the expected tangy, acidic flavour as well as colour of a soft drink. Additionally, a higher pH can increase susceptibility to microbial spoilage as well as decrease the effectiveness of preservatives (i.e. sorbic and benzoic acid) and therefore measures such as pasteurisation or UHT need to be considered (Ashurst et al., 2017).

Soft tribology analysis provides insight into the mouthfeel of oral processing of foods and beverages. Higher frictional coefficients are generally associated with increased surface roughness and sensations such as astringency or dryness, while lower friction reflects smoother lubrication behaviour (Corvera-Paredes et al., 2022). This method has been widely applied to beverages such as beer, wine, tea and soft drinks (Chong et al., 2019; Fox et al., 2022; Laguna

& Sarkar, 2017; Steinbach et al., 2014). In this study, ELF showed higher lubricating properties than EDF. These results are consistent with a previous study by Ahern et al. (2025) that reports that the presence of free amino acids and organic acids can influence frictional properties, and ELF showed reduced metabolite concentration compared to EDF (Laguna & Sarkar, 2017; Upadhyayet al., 2016). Compared with their controls, NAB and AJ showed elevated frictional factors at the boundary regime believed to be associated with astringency (Prakash et al., 2013). Comparable findings have been reported for other NABs, where high friction corresponds with sensory descriptors of thin or watery mouthfeel relative to alcoholic ones described as creamy and smooth (Fox et al., 2022). LL Free had higher frictional properties compared to LL with added sugar. The addition of EDF or ELF decreased frictional factors, therefore may sensorially decrease astringency and increase the viscosity of the mouthfeel. Contrary to expectations, BT and IT, typically perceived as astringent, exhibited the opposite tribological behaviour with the highest lubricating properties, even in control samples with no EP addition. One explanation is that the addition of protein may facilitate the formation of a thin, lubricating interfacial layer, improving sliding between the ball and pin, as previously proposed by Kew et al. (2021). Although polyphenols generally increase oral friction by forming precipitating complexes with proteins, the behaviour of BT and IT suggests that the nature of protein–polyphenol interactions depends on factors such as molecular size, charge, tannin flexibility and protein conformation (Alu'datt et al., 2018, Rana et al., 2022). For example, large protein–polyphenol aggregates can form lubricating films (Chojnicka et al., 2008) while tea types also differ in polyphenol composition, with green tea typically containing higher polyphenol levels than black tea (Rana et al., 2022). However, while this study shows IT exhibiting smaller particle sizes (~350 nm) and BT showing larger particle sizes (~2000 nm), the literature shows conflicting findings, with studies reporting that mainly smaller particle sizes have reduced frictional parameters (Corvera-Paredes et al., 2022; Kew et al., 2021). However, the tests in the current study were conducted without the addition of saliva which would further influence the lubrication behaviour under real oral conditions. Therefore, this discrepancy needs to be considered and highlights how a combination of factors can be involved in tribological behaviour.

5.5. Conclusion

Several beverage systems were evaluated following the addition of EDF and ELF at various concentrations. Clear differences were found depending on both the concentration of protein

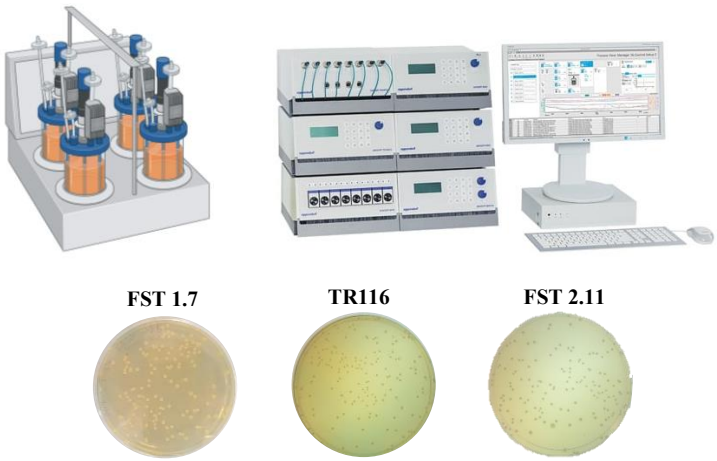
added, and the type of beverage system studied. Increased protein concentration elevated the pH across all beverage systems, leading to a trend whereby beverages became more stable and clearer at higher protein levels due to changes in solubility as a result of shifts in their isoelectric points. Moreover, protein inclusion increased both viscosity and liquid density accordingly. Lubricating properties increased with the addition of EP ingredients in all beverages, with ELF showing more effective lubrication in all beverage systems. Beverages with polyphenols, such as BT and IT, demonstrated the lowest frictional parameters regardless of protein inclusion level. Incorporating both EP ingredients into these beverages enhanced their protein content by offering a convenient ready-to-drink protein soft drink, while also promoting sustainability benefits through the use of upcycled proteins. Nevertheless, the current study highlights the need for further research to address the impact of protein addition, particularly plant-based proteins, on the stability and quality of these beverages. No single beverage system demonstrated a clear advantage over another and it should be noted that protein inclusion can potentially compromise sensory properties, which should be carefully considered during formulation. Future work should investigate the antioxidant properties of these ingredients and their interactions within the beverage systems including polyphenols, particularly in tea based beverages. Furthermore, trials to ensure microbial stability are essential for safe consumption, especially in ready-to-drink soft drinks. Continued research is key for advancing the development of nutritious, stable, safe, consumer accepted protein fortified beverages.

5.6. Acknowledgements

The authors would like to thank EverGrain Ingredients for kindly providing EDF and ELF ingredients.

6. Chapter 6

BSG-derived protein isolate as a base for fermented protein beverages: modulation of techno-functional and sensory characteristics using different lactic acid bacteria strains



Published in Food Chemistry Advances: Ahern, N., Nyhan, L., Coffey, A., O'Riordan, P., Arendt, E. K., & Sahin, A. W. (2026). BSG-derived protein isolate as a base for fermented protein beverages – modulation of techno-functional and sensory characteristics using different lactic acid bacteria strains. *Food Chemistry Advances*, *11*. https://doi.org/10.1016/j.focha.2026.101314

Abstract

Brewers' spent grain is the most abundant by-product during the brewing process and can consist of barley and adjuncts, such as rice. The isolated protein fraction is suitable for beverage application since it has a high solubility in water. However, challenges of its use in novel beverages include poor techno-functional properties, and an unfavourable sensory profile often causing astringency and off-flavours. The aim of this study was to investigate the use of lactic acid bacteria (LAB) fermentation for modulation of the techno-functional and sensory properties of EverPro™ Light fraction (ELF), a barley and rice protein isolate, in a simple beverage system. The beverages were formulated with 10% (w/v) ELF and 5% (w/v) sucrose, followed by single-strain fermentation using *Lactiplantibacillus plantarum* FST 1.7, *Leuconostoc citreum* TR116 and *Lactobacillus amylovorus* FST 2.11. First, the fermentation performance was evaluated by determining the pH, total titratable acidity (TTA), viable cell counts, sugars and acids during fermentation. TR116 showed the fastest growth, while FST 1.7 and FST 2.11 resulted in the highest acidification. Secondly, the techno-functionality of the fermented beverages was investigated. Compared to the control (non-fermented), the changes in techno-functionality of the fermented beverages were strain-specific, showing darker colour, increased viscosity, turbidity and separation rate. Fermentation with FST 2.11 resulted in increased friction during tribological measurements, while FST 1.7 and TR116 enhanced lubrication. Sensory analysis revealed that fermentation with FST 1.7 imparted fruity flavours, whereas FST 2.11 produced dairy-like notes. TR116 showed lowest malty/cereal flavour. These fermented protein-based beverages showed improved organoleptic properties supporting the potential of ELF as a substrate to produce high plant protein soft drinks

6.1. Introduction

Fermentation is a widely used processing technique that can enhance nutritional, sensorial and functional properties of food and beverages (Hidalgo-Fuentes et al., 2024). Lactic acid bacteria (LAB) fermentation has been a long-used strategy serving a crucial role in transforming diverse substrates into value added products. Well known fermented products involving LABs include the fermentation of milk to yoghurt, kefir and cheese, cereals to create sourdough bread, soya beans into tofu and tempeh, as well as fermenting various types of vegetables to yield kimchi, sauerkraut and more. These fermented products allow for the development of unique flavours and extended shelf life but also offer health benefits (Binda & Ouwehand, 2019; Gänzle & Salovaara, 2019; Park & Kim, 2019). Water based, non-alcoholic fermented beverages utilising LAB include water kefir (tibicos) produced by fermenting a sucrose solution usually with dried fruits, as well as kombucha, which is fermented from sweetened tea. Both products are fermented using a symbiotic culture of bacteria and yeast embedded in a polysaccharide matrix (Lynch et al., 2021; Nyhan et al., 2022). These beverages have gained popularity due to their potential to support gut health by providing probiotics and bioactive fermented byproducts, for overall wellbeing. Pasteurisation is sometimes employed post-fermentation to prevent the risk of continued fermentation and an increase in ethanol levels, which then inactivates the health benefits of the probiotic live bacteria (Cufaoglu & Erdinc, 2023; Lynch et al., 2021; Nyhan et al., 2022). However, emerging evidence indicates that LAB fermentation has shown to produce postbiotics, non-viable microbes or their metabolites, that confer health benefits similar to probiotics without requiring the microbes to be alive (Chang et al., 2021; Żółkiewicz et al., 2020).

As global dietary patterns increasingly shift towards sustainability, fermentation offers a promising approach to enhance functionality and value to novel protein products. The most recent EAT- Lancet commission report emphasises that shifting towards more plant-forward dietary patterns, alongside the upcycling of food system side streams, is essential for achieving both nutritional and global health targets (Rockström et al., 2025). LAB fermentation has been applied in beverages across a variety of protein matrixes for different purposes. For example, the fermentation of a casein or whey protein beverage with LAB has been shown to improve digestibility and increase phenolic compounds (Alrosan et al., 2024; Cho et al., 2015). Comparable benefits were reported for a tofu byproduct (liquid fraction) beverage, showing additional enhanced antioxidant activity and sensory properties (Azi et al., 2020; Tu et al.,

2019). Manus et al. (2021) further demonstrated that fermenting combinations of pea and rice proteins, as well as pea and hemp proteins, with LABs (including *Lactobacillus acidophilus, Lactobacillus casei and Lactobacillus rhamnosus*) can yield probiotic drinks with increased digestibility and viscosity. While these studies, along with recent reviews such as Hidalgo-Fuentes et al. (2024), have summarised advances in plant-based beverage fermentation, most focus on formulations intended as milk alternatives, whereas fewer studies have addressed the fermentation of high protein plant-based beverages to produce soft drinks.

The LAB strains *Lactiplantibacillus plantarum* FST 1.7, *Leuconostoc citreum* TR116 and *Lactobacillus amylovorus* FST 2.11 have been applied in various fermentation studies targeting sensory enhancement and improvement of ingredient functionality for application in variety of food and beverage products. These strains have demonstrated benefits across multiple substrates, including non-alcoholic sour beer (FST 1.7, FST 2.11) (Nyhan et al., 2023; Peyer et al., 2017), brewing byproducts including rootlets (FST 1.7, TR116, FST 2.11) (Neylon, et al., 2023) and brewers spent yeast (FST 2.11) (Jaeger et al., 2024), lentil-based yoghurt alternatives (TR116) (Boeck et al., 2022), sugar-reduced burger buns (TR116) (Sahin et al., 2019), pâté-based products (TR116) (Sahin et al., 2024) and high-protein faba bean ingredients (Hoehnel et al., 2020). EverPro™ Light Fraction (ELF) is a decolourised BRPI manufactured by the company EverGrain (Turck et al., 2023). This ingredient exhibits high solubility across a wide pH range, including acidic conditions, and has been shown to enhance appearance and improve sensory characteristics by reducing off flavours (bitter and burn/roasty descriptors) and minimising aroma compounds, making it more adaptable for beverage formulations (Ahern et al., 2025). This makes ELF a promising substate for further exploration in producing high protein fermented beverages, positioning it as a promising ingredient for non-alcoholic functional beverage formulations.

The aim of this study was 1) to evaluate the fermentation performance of three different LAB strains in a simple ELF-based beverage system containing a high protein content, which included monitoring pH, total titratable acidity (TTA), viable cell count, sugar and acid profile, over 48 hrs of fermentation in 24 hrs intervals; and 2) to investigate changes in techno-functionality and tribology of the beverages, and their sensory characteristics after 48 hrs of fermentation.

6.2. Materials and methods

6.2.1. Materials

Barley and rice protein isolate, EverPro™ Light Fraction (ELF) was obtained from EverGrain Ingredients (St. Louis, MO, USA). All chemicals were purchased from Sigma- Aldrich (St. Louis, MO, USA) unless stated otherwise. Prior to fermentation, the presence of microbial spore formers in ELF, common contaminants in plant-based protein extracts that can affect food safety and LAB fermentations, was assessed by Eurofins Food Testing Ltd (Dungarvan, Waterford, Ireland) ELF was fermented with *Lactiplantibacillus plantarum* FST 1.7 and *Lactobacillus amylovorus* FST 2.11, which belong to the culture collection stock of the Cereal and Beverage Science Research Group in the School of Food and Nutritional Sciences Department, University College Cork, Ireland, and *Leuconostoc citreum* TR116, which belongs to the culture collection of the Department of Biological Sciences of Munster Technological University Ireland (**Table 6.1)**. These LAB strains were maintained as stock cultures and were stored frozen in 40% glycerol at – 80°C. The strains were subcultured routinely on deMan Rogosa Sharpe (MRS) agar plates supplemented with 0.05 g/L bromocresol green, under anaerobic conditions using AnaeroGen gas packs (Thermo Fisher Scientific, Walham, MA, USA) at 30 °C for 48 hrs.

Table 6.1: Attributes of the lactic acid bacteria used for experimental analysis.

Species	***Lactiplantibacillus plantarum***	***Leuconostoc citreum***	***Lactobacillus amylovorus***
Strain	FST 1.7	TR116	FST 2.11
Metabolism	Heterofermentative	Heterofermentative	Homofermentative
Fermentation substrate	Sucrose	Sucrose	Sucrose
Source	Malted barley	Yellow pea sourdough	Brewing environment
Special traits	Antifungal producer, high acid producer	Mannitol producer, antifungal producer	Antimicrobial producer, high acid producer
References	(Dal Bello et al., 2007)	(Rice et al., 2020; Sahin et al., 2021)	(Peyer et al., 2017)

6.2.2. Beverage Fermentation

Prior to fermentation, cell suspensions of each LAB strain were prepared by inoculating a single colony from a fresh agar plate into 10 ml of MRS broth and incubating at 30°C for 24 hrs. This was followed by subculturing 1% (v/v) of the inoculum into 50 ml of fresh MRS broth and incubating at 30°C for 18 hrs. Cells were harvested by centrifugation at 4863 rcf for 5 mins

at 4°C. The supernatant was discarded, and the cell pellet was resuspended in 50 ml of sterile tap water. Cells were washed twice by repeating the centrifugation and resuspension steps. ELF beverage fermentations were performed in 1 L batch bioreactor vessels on the DASGIP Bioblock combined with the DASGIP TC4SC4 and DASGIP PH8 modules (Eppendorf, Stevenage, UK) for temperature and agitation control. An 800 ml solution consisting of 10% (w/v) ELF and 5% (w/v) sucrose was prepared with sterilised tap water and placed in each bioreactor, providing approximately 8 – 9 g of protein per 100 ml. The ELF-based beverage was sterilised by heating to 90°C for 30 mins and cooled down to 30°C and sterility was confirmed by plating on plate count agar and incubating at 30°C for 48 hrs. For the fermentations, LAB strains were inoculated at 1% (v/v) into to the ELF-based beverage to obtain an average initial cell concentration of 10^7 CFU/ml. Fermentations were carried out at 30 °C with constant mixing at 50 rpm for 48 hrs. Aliquots were taken in duplicate from each bioreactor at 0, 24 and 48 hrs to analyse pH, TTA, viable cell counts, sugars and organic acids. After 48 hrs, the fermented beverages were pasteurised at 72°C for 15 mins, and aliquots of the beverage solutions were frozen at - 20°C for subsequent analysis. Pasteurised ELF beverage solution (unfermented ELF beverage) was used as a control.

6.2.3. Microbial Growth

Viable cell counts were determined by serial diluting 1 ml of the fermented ELF beverage in 9 ml of Ringer's solution, followed and plating 0.1 ml of each dilution onto MRS agar supplemented with 0.05 g/L bromocresol green. Plates were incubated anaerobically using AnaeroGen gas packs at 30°C for 48 hrs and counted to determine CFU/ml (colony forming units). Additionally, the growth rate of each LAB was calculated as the slope of the line between 0 to 24 hours and 24 to 48 hours expressed in Log CFU/ml per hour.

6.2.4. pH and Total Titratable Acidity

pH was measured using a pH meter (Mettler Toledo, OH, USA) at 20°C. TTA was determined using the method described by Nyhan et al. (2023), where 20 ml of beverage sample was titrated against 0.1 M NaOH until a neutral pH value of 7 was reached, followed by a 1-minute waiting period. TTA was expressed as the amount of 0.1 M NaOH/ml sample. If the pH of the beverage was above 7, only pH was recorded and TTA was considered as non- applicable (n.a).

6.2.5. Quantification of Sugars and Organic Acids

Sugars and organic acids were extracted according to Hoehnel et al. (2020). Briefly, 2 g of sample was added to 15 ml of 80% EtOH (ethanol) at 80°C. The samples were then sonicated

at an amplitude of 100% for 30 secs using an ultrasonic homogeniser (Bandelin, Sonoplus), and centrifuged at 1800 rcf for 10 mins. The resulting supernatant was decanted into a new tube. To ensure complete extraction of sugars and organic acids, the pellet was washed again with 80% EtOH at 80°C, sonicated and centrifuged using the same conditions mentioned above, and the two supernatants were combined. A vacuum centrifuge (Scanvac, Lynge, Denmark) was used to evaporate the EtOH and concentrate the extracts (2000 rpm, 55 °C, 3.5 hrs) before the final volume was adjusted to 10 ml with NaN_3 (sodium azide) solution (50 ppm). The obtained extracts were filtered twice, first through a syringe-driven filters (Corning, NY, USA) with a pore size of 0.45 µm, followed by 0.2 µm filter. The samples were diluted and stored frozen until chromatographic analysis. The quantification of selected mono, di, oligo-saccharides and polyols was carried out as previously described by Ispiryan et al. (2019) with a Dionex ICS-5000+ system (Sunnyvale, CA, USA) and pulsed amperometric detection. An external calibration was used for each of the compounds (0.05–20 ppm) and the separation was performed on column: Thermo Scientific Dionex CarboPac PA1 column (2×250 mm; Thermo Fisher Scientific, Waltham, MA, USA; used for quantification of sucrose, glucose, fructose, mannitol. The column was equipped with the matching guard column. A flow rate of 0.25 ml/min was chosen and eluents and elution conditions (isocratic/gradient) were set as reported by Ispiryan et al. (2019). The separation of organic acids (acetic and lactic acid) was performed using a Dionex UltiMate 3000 System (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Hi-Plex H column (7.7×300 mm; Agilent Technologies, Santa Clara, CA, USA) and the matching guard column, which was operated at a column temperature of 60 °C and with 5 mM sulphuric acid as eluent. The compounds were quantified using an external calibration (0.03-6 g/L) and an ultraviolet light/diode array detector (UV/DAD; Thermo Fisher Scientific, Waltham, MA, USA;UV spectra recorded between 190 and 400 nm; quantification at 210 nm

6.2.6. Colour

The CIE L* a* b* and RGB measurements (refers to the image where each pixel is defined by the amount of red, green, and blue colours) of the samples were measured with a UV-VIS spectrophotometer (Thermo Scientific Genesys 50, Waltham, MA, USA) using the method described by Delgado-González et al. (2018), calculated according to CIELab parameters at illuminant D65. Briefly, the colour coordinates of the beverages were determined by transmittance spectrum of the sample in the range of 380 - 780 nm. The spectrum was recorded

with a wavelength resolution in 5 nm steps between each measurement and blanked with distilled water.

6.2.7. Turbidity

Turbidity was measured using a turbidity meter (Thermo Scientific Eutech TN-100 Waterproof Turbidimeter, Singapore). Samples were measured at 20°C. The turbidity meter was calibrated and a sample volume of 10 ml was used for analysis ranging from 0 - 2000 nephelometric turbidity unit (NTU) with a wavelength of 850 nm.

6.2.8. Apparent Viscosity

Rheological behaviour was determined using a rheometer (MCR301, Anton Paar, Graz, GmbH, Austria) equipped with a concentric cylinder measuring attachment (Anton Paar, GmbH, Graz, Austria) as described by Ahern et al. (2023). The dynamic viscosity (mPa·s) was measured as a function of shear rate ranging from 0.5 to 100 (1/s) at 20°C. Then the apparent viscosity at 80.7 (1/s) was evaluated and used to compare the beverages with each other.

6.2.9. Stability

Stability was determined using an analytical centrifuge (LUMiSizer, LUM GmbH, Berlin, Germany), through phase separation based on light transmission during centrifugation. The cycle configuration involved taking 100 profiles in 10 s intervals centrifugated at 1000 rcf, at a wavelength of 865 nm and a temperature of 20°C. During centrifugation, the samples were illuminated with near infrared light and transmitted light was detected by sensors across the entire sample. The percentage of integrated light transmission increased over time which is then calculated by the software (percentage of integrated light transmission, plotted against time where the slope of the line to fit this curve) and the separation rate in % transmission is given per minute as reported by Vogelsang-O'Dwyer et al. (2021).

6.2.10. Tribology

Tribological measurements were conducted on an MCR301 rheometer using the BC-12.7 ball-on-3-pins tribology attachment (Anton Paar GmbH, Graz, Austria) at 37°C as described by Fox et al. (2022). The attachment contains changeable tribopairs (a glass ball and polydimethylsiloxane (PDMS) pins), which were supplied by Anton Paar (Graz, Austria). New pins were used for each test consisting of one run-in period and two repeated measurements of Stribeck curves. Before starting a test, the pin-holder and ball-holder were washed gently using a dilute detergent solution and rinsed thoroughly as well as being rinsed in acetone and dried

with lab-wipes with the tribopairs. The test sequence was as followed: first the glass ball was lowered until a target force of 3 N was reached and held at that height for an equilibration period of 1 min. Furthermore, the following settings of the measurement system were selected: a logarithmic increase in sliding speed from 10^{-8} to 1 m/s was performed recording 80 data points with point duration from 1 to 10 seconds logarithmic, followed by a resting period of 1 min. The control and the fermented beverages were measured using 1 ml aliquots. Three consecutive Stribeck curve measurements were performed for each sample. The first measurement served as a run-in period and was excluded from the data set. Each sample was performed in triplicate, resulting in six evaluated datasets per sample.

6.2.11. Descriptive Sensory Analysis

Prior to conducting sensory analysis, all four samples were sent to Eurofins Food Testing Ltd (Dungarvan, Waterford, Ireland) for microbial testing. The lab examined the samples for various food borne pathogens including *Listeria* species (including *L. monocytogenes*), *Salmonella*, *E. coli*, Coagulase Positive *Staphylococcus* and presumptive *Bacillus cereus*. All pathogens were either not detected or within the satisfactory limit (< 10 CFU/ml), indicating compliance with the microbiological criteria for ready to eat products as defined by Commission Regulation (EC) No 2073/2005 (European Parliament & Council, 2005).

Sensory analysis of the unfermented control and the fermented ELF beverages were conducted by a panel of experienced sensory testers (n= 7, 2 male and 5 female, age range 23 - 32) recruited from the School of Food and Nutritional Sciences at University College Cork. The panellists evaluated the intensity of the following descriptors on a scale of 0-10, with 10 indicating highly detectable (extreme) and 0 indicating undetectable (none): taste: sweet and sour; flavour: honey, fruity, citrus, earthy/muddy, dairy; mouthfeel: viscosity. The definitions of the descriptors are displayed in **Supplementary Table 6.1**. The sensory analysis took place in a sensory analysis room and was completed in duplicate across two separate sessions.

6.2.12. Statistical analysis

All analysis was performed in triplicate unless stated otherwise. All statistical analysis was performed using IBM SPSS version 29 (Armonk, NY, USA). A one-way ANOVA with post-hoc Tukey test (p value ≤ 0.05) was completed to identify significant differences among groups. In cases where equal variances were not assumed, a correction using Games Howell (p value ≤ 0.05) post hoc test was applied to the data. When data was not normally distributed, a non-parametric Kruskal Wallis test value (p value ≤ 0.05) was used.

6.3. Results

The results reveal firstly the fermentation performance of the three different LAB strains using the ELF-based protein beverage as a substrate and secondly the quality differences regarding techno-functionality and sensory between the fermented beverages.

6.3.1. Fermentation performance

To evaluate the fermentation performance of the different strains, changes in pH and TTA, as well as microbial growth kinetics were determined in 24 hrs intervals for 48 hrs. Additionally, changes in sugars and acids during fermentation reveals insights in metabolic traits.

6.3.1.1. pH and TTA

Figure 6.1A exhibits the changes in pH during single-strain fermentations with *L. plantarum* FST 1.7, *L. citreum* TR116 and *L. amylovorus* FST 2.11, while the TTA results are shown in **Table 6.2**. The unfermented control, as well as the fermented beverages, showed an average pH of ~8 at T0. Both FST 1.7 and FST 2.11 caused a similar pH reductions after 24 hrs (at 7.53 ± 0.07 and 7.37 ± 0.08 respectively), followed by a steep decline after 48 hrs (at 5.42 ± 0.22 and 5.47 ± 0.16 respectively). Fermentation with TR116 resulted in a pH drop from pH 7 after 24 hrs to 5.88 after 48 hrs. TTA values were only recorded at time point 48 hrs as the beverages had a pH ≥ 7 at time points 0 hrs and 24 hrs. FST 1.7 showed the highest TTA (0.26 ± 0.03 of 0.1M NaOH/ml), followed by FST 2.11 (0.20 ± 0.03 of 0.1M NaOH/ml). TR116 showed the lowest acidity (0.14 ± 0.01). Overall, the increase in TTA corresponded with the decrease in pH.

Table 6.2: Fermentation performance of unfermented control and fermented ELF-based beverages including *Lactiplantibacillus plantarum* FST 1.7, *Leuconostoc citreum* TR116 and *Lactobacillus amylovorus* FST 2.11. Data are expressed as mean ± standard deviation. Values in the same row that share a letter do not differ significantly ($p < 0.05$). n.a = not applicable.

	Control	FST 1.7	TR116	FST 2.11
TTA 48h (ml 0.1 M NaOH/ml)	n.a	0.26 ± 0.03 [a]	0.14 ± 0.01 [b]	0.20 ± 0.03 [c]
Growth rate 0 to 24 h (Log CFU/ml per hr)	n.a	0.030 ± 0.01 [a]	0.073 ± 0.02 [b]	0.020 ± 0.00 [a]
Growth rate 24 to 48 h (Log CFU/ml per hr)	n.a	0.035 ± 0.02 [a]	0.022 ± 0.01 [ab]	0.018 ±0.01 [b]

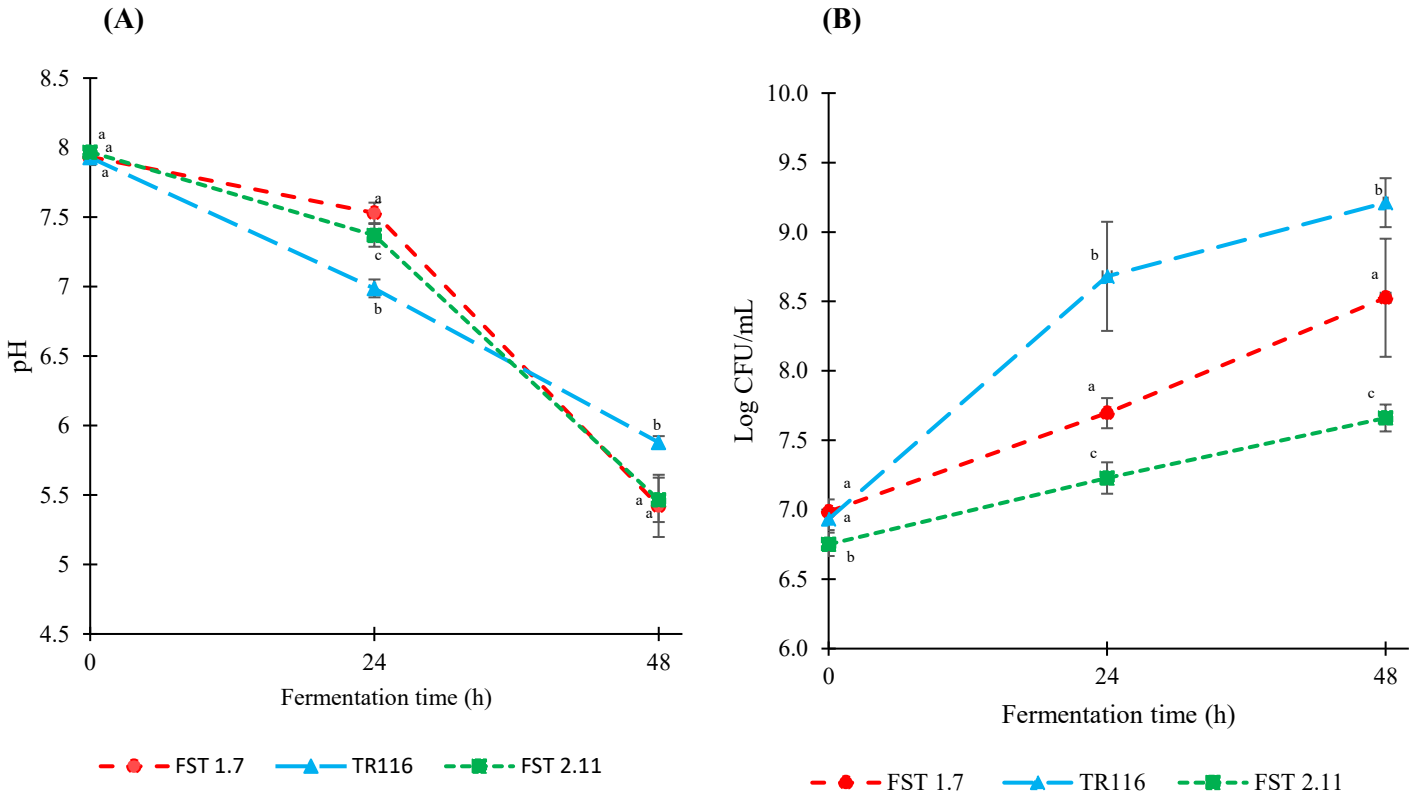


Figure 6.1: (A) Acidification and (B) microbial growth at times 0, 24 and 48 hr fermentation of fermented ELF beverages including *Lactiplantibacillus plantarum* FST 1.7, *Leuconostoc citreum* TR116 and *Lactobacillus amylovorus* FST 2.11. Points which share the same letter do not differ significantly ($p < 0.05$).

6.3.1.2. Microbial growth kinetics

The initial cell counts for strains FST 1.7, TR116 and FST 2.11 are displayed in **Figure 6.1B**, and were 6.99 ± 0.09, 6.93 ± 0.08 and 6.75 ± 0.08 log CFU/ml respectively, increasing to maximum cell counts of 8.53 ± 0.43, 9.21 ± 0.18 and 7.66 ± 0.10 log CFU/ml after 48 hrs. TR116 reached the highest cell count after 24 hrs, with a significantly higher growth rate between 0 and 24 hrs (0.073 ± 0.02 log CFU/ml per hour), as shown in **Table 6.2**, which declined between 24 and 48 hrs (0.022 ± 0.01 log CFU/ml per hour). In contrast, FST 1.7 exhibited a notable increase in growth rate between 24 and 48 hours, whereas FST 2.11 maintained relatively stable growth, with the lowest viable cell count throughout.

6.3.1.3. Sugar and acid profiles

The sugar profiles, (monosaccharides, disaccharides and polyols), along with the organic acids of each fermented beverage were analysed over 48 hrs in 24 hrs intervals. The profiles are illustrated in **Figure 6.2**, (values are presented in **Supplementary Table 6.3**). At 0 hrs, all beverages contained ~5% sucrose. After 24 hrs fermentation, sucrose levels remained largely unchanged with no significant reduction observed in beverages fermented with FST 1.7 and

TR116, whereas FST 2.11 caused a notable decrease to 4.75 ± 0.12 g/100ml. By 48 hrs, a significant decrease in sucrose was detected across all beverages with the most pronounced decrease observed with FST 2.11 (2.87 ± 0.51 g/100ml), followed by TR116 (4.36 ± 0.14 g/100ml) and FST 1.7 (4.55 ± 0.33 g/100ml). Fermentation with FST 2.11 resulted in a significantly higher glucose level (1.15 ± 0.34 g/100ml) after 48 hrs compared to FST 1.7 and TR116. ELF-based beverages fermented by FST 1.7 showed the lowest quantity of fructose after fermentation (0.07 ± 0.02 g/100ml), followed by FST 2.11 (0.14 ± 0.04 g/100ml) and TR116 (0.17 ± 0.02 g/100ml). Notably, TR116 was the only strain to produce mannitol, reaching 0.58 ± 0.04 g/100ml after the 48 hrs.

Organic acid production also varied between strains. Both FST 1.7 and FST 2.11 generated significantly higher amounts of lactic acid (0.31 ± 0.04 g/10 ml and 0.31 ± 0.06 g/100ml, respectively) compared to TR116 (0.14 ± 0.01 g/100ml).

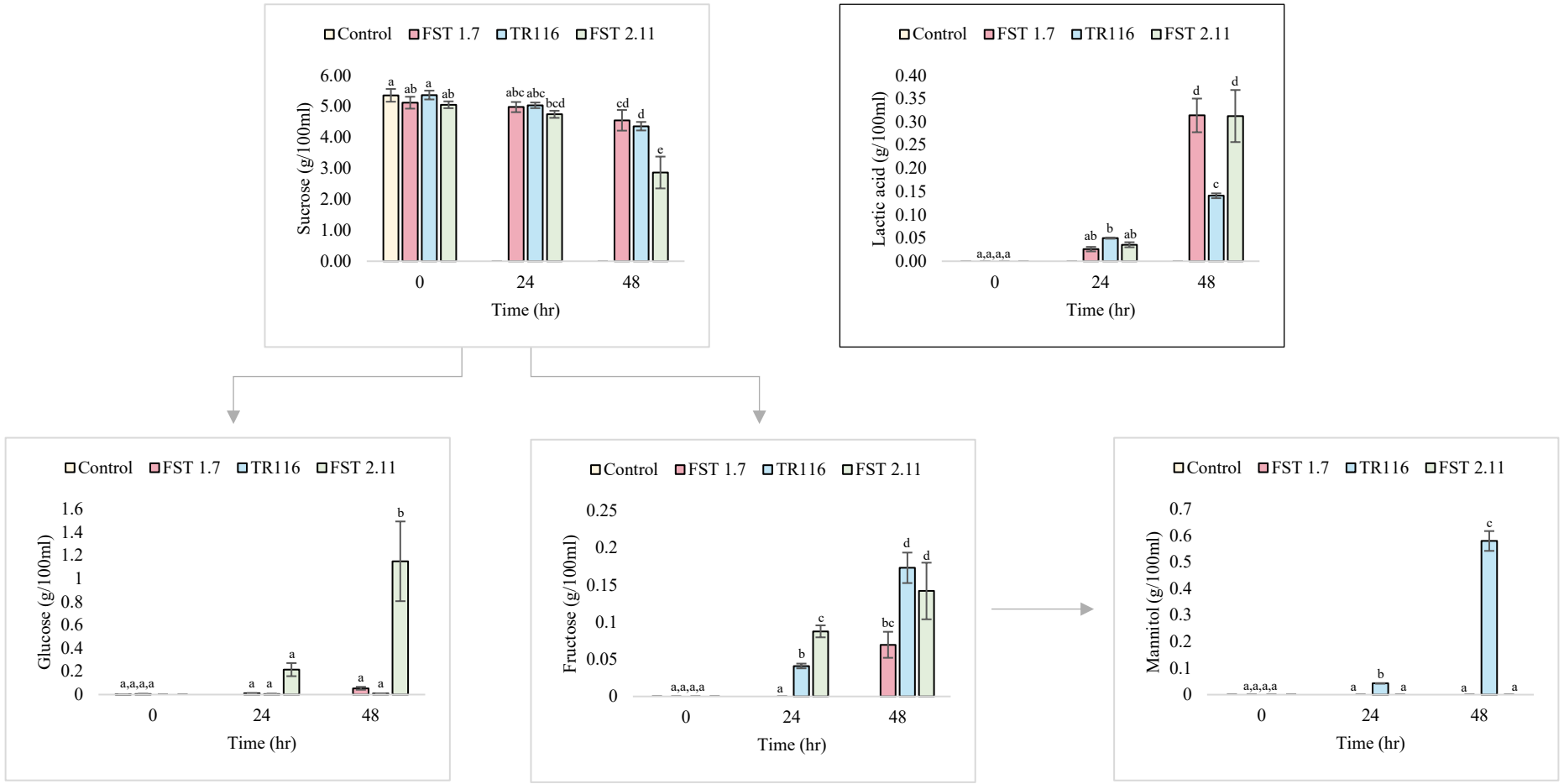


Figure 6.2: Profile of sugars and acids at times 0 hrs, 24 hrs and 48 hrs fermentation expressed in g/100ml of unfermented control and fermented ELF beverages including *Lactiplantibacillus plantarum* FST 1.7, *Leuconostoc citreum* TR116 and *Lactobacillus amylovorus* FST 2.11. Points which share the same letter do not differ significantly ($p < 0.05$).

6.3.2. Techno-functional properties of fermented beverages

Table 6.3: Techno-functional properties of unfermented control and fermented ELF-based beverages including *Lactiplantibacillus plantarum* FST 1.7, *Leuconostoc citreum* TR116 and *Lactobacillus amylovorus* FST 2.11. Data are expressed as mean ± standard deviation. Values in the same row that share a letter do not differ significantly ($p < 0.05$). n.a = not applicable.

	Control	FST 1.7	TR116	FST 2.11
Colour L* value	22.38 ± 0.11 ^{a}	12.40 ± 0.44 ^{b}	13.65 ± 0.19 ^{c}	11.74 ± 2.90 ^{bc}
Colour a* value	17.05 ± 0.14 ^{a}	14.60 ± 0.25 ^{b}	14.36 ± 0.17 ^{b}	15.56 ± 1.30 ^{ab}
Colour b* value	91.88 ± 0.17 ^{a}	75.99 ± 0.73 ^{b}	78.02 ± 0.33 ^{c}	74.95 ± 4.81 ^{bc}
Turbidity (NTU)	555 ± 8 ^{a}	1180 ± 109 ^{b}	608 ± 43 ^{a}	868 ± 100 ^{c}
Separation rate (%/min)	0.40 ± 0.06 ^{a}	1.27 ± 0.10 ^{b}	0.97 ± 0.03 ^{c}	0.67 ± 0.08 ^{d}
Apparent viscosity at 80 1/s (mPa·s)	3.38 ± 0.20 ^{a}	4.36 ± 0.65 ^{b}	3.92 ± 0.32 ^{b}	5.12 ± 0.84 ^{b}

6.3.2.4. Colour and Turbidity

The colour and turbidity values of the beverages are presented in **Table 6.3**, as well as images and RGB images of the unfermented and fermented beverages are shown in **Figure 6.3**. All fermented beverages exhibited lower L* values than the control (unfermented ELF-based beverage) at 22.38 ± 0.1. Among the fermented beverages, L* values were comparable at 13.65 ± 0.19 (TR116), 12.40 ± 0.44 (FST 1.7) and 11.74 ± 2.90 (FST 2.11). Fermented beverages resulted in significantly higher turbidity results compared to unfermented control (555.17 ± 8.01 NTU) with the exception of TR116 (608.33 ± 43.06 NTU). Beverages fermented with FST 1.7 showed the highest turbidity at 1180.67 ± 109.75 NTU, followed by FST 2.11 at 868.33 ± 100.05 NTU.

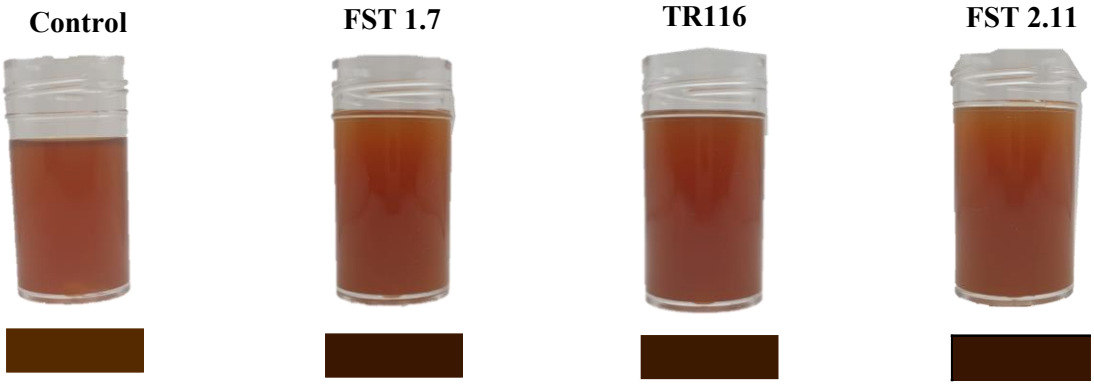


Figure 6.3: Images and RGB pictures of unfermented control and fermented ELF-based beverages including *Lactiplantibacillus plantarum* FST 1.7, *Leuconostoc citreum* TR116 and *Lactobacillus amylovorus* FST 2.11.

6.3.2.5. Dispersion Stability

The separation rate of each sample is displayed in **Table 6.3**. The unfermented control showed a significantly lower separation rate (0.40 ± 0.06 %/min) compared to all fermented beverages. Among the fermented beverages FST 2.11 resulted in the highest dispersion stability (0.67 ± 0.08 %/min) compared to TR116 (0.97 ± 0.03 %/min) and FST 1.7 (1.27 ± 0.10 %/min) which showed the highest separation rate. Lumisizer graphs shown in **Figure 6.4** illustrate sedimentation in protein beverages fermented by FST 1.7 and FST 2.11 as evidenced by the accumulation of red and green lines towards the end of the transmission profile compared to TR116 and the control showing little to no sedimentation.

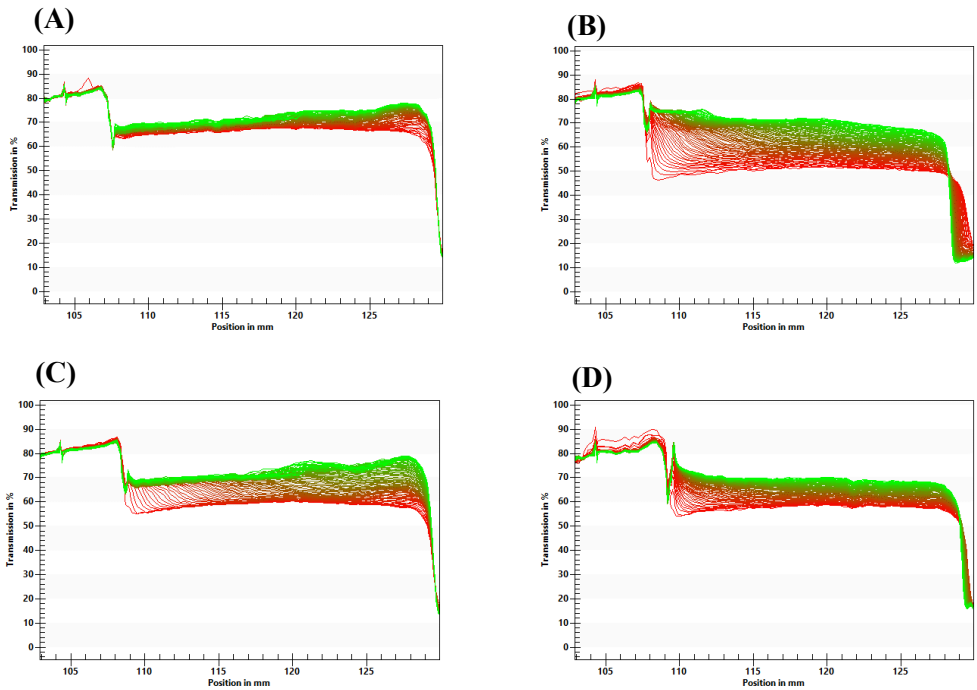


Figure 6.4: Lumisizer graphs showing the transmission profiles of (A) fermented control and fermented ELF-based beverages including (B) *Lactiplantibacillus plantarum* FST 1.7, (C) *Leuconostoc citreum* TR116 and (D) *Lactobacillus amylovorus* FST 2.11 of near infrared light as a function of position. The top of the samples begins at the left-hand side of the graph. The first transmission is shown in red and the last one shown in green.

6.3.2.6. Apparent Viscosity and Tribology

Viscosity and frictional properties are factors that can serve as important functional attributes capable of influencing the mouthfeel of beverages. The unfermented control exhibited the lowest viscosity (3.38 ± 0.20 mPa·s) whereas the fermented beverages showed significantly higher values. Among the strains, FST 2.11 caused the highest viscosity, followed by FST 1.7 and TR116 (**Table 6.3**), suggesting the changes in viscosity are strain dependent.

Stribeck curves displayed in **Figure 6.5**, indicate distinct lubrication behaviours among the samples. Beverages fermented by FST 2.11 exhibited the lowest lubrication properties overall. Fermentation with TR116 demonstrated beverages with the highest lubrication properties, with significant differences observed at sliding speeds 10^{-2} m/s and 1 m/s (**Supplementary Table 6.4**). At sliding speeds ranging from 10^{-8} m/s to 10^{-3} m/s, the unfermented control and beverages fermented by FST 1.7 exhibited similar Stribeck profiles. However, between 10^{-3} m/s and 10^{-1} m/s, the lubrication of FST 1.7-fermented protein beverages deceased, whereas it increased in the unfermented control.

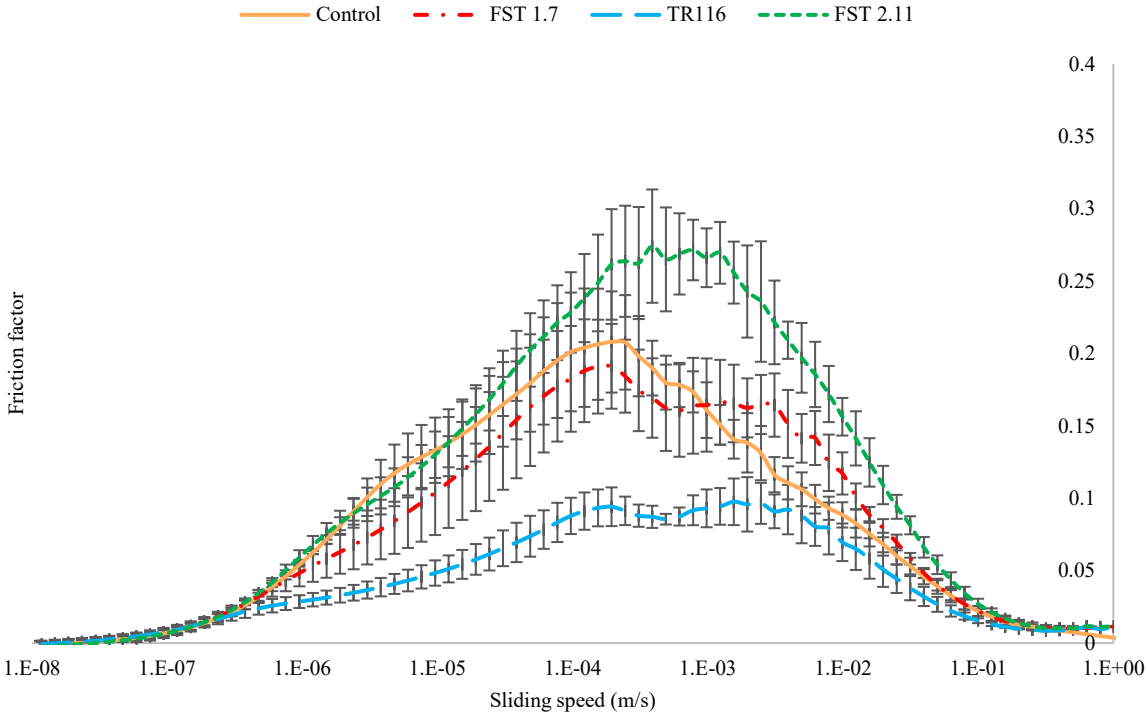


Figure 6.5: Stribeck curves of unfermented control and fermented ELF beverages including *Lactiplantibacillus plantarum* FST 1.7, *Leuconostoc citreum* TR116 and *Lactobacillus amylovorus* FST 2.11 with friction factor as a function of sliding speed (m/s).

6.3.3. Descriptive Sensory Analysis of fermented beverages

The sensory evaluation revealed clear strain dependent differences in flavour and mouthfeel attributes after the 48 hrs fermentation shown in **Supplementary Table 6.5** and **Figure 6.6**. No significant differences were found in the overall taste attributes (sour, sweet) of the beverages.

Protein beverages fermented by FST 1.7 did not differ significantly in honey flavour compared to the unfermented control, whereas beverages fermented by TR116 and by FST 2.11 were

rated as having significantly less honey flavour. However, the beverage fermented by FST 1.7 was distinguished by exhibiting a significantly elevated fruity flavour compared to all other strains. Beverages fermented by TR116 exhibited the lowest malty/cereal flavour intensity, which was significantly lower than that of the unfermented control and FST 2.11 fermented beverages but not to FST 1.7.

All beverages were rated as very low in citrus flavour intensity, with scores ranging from 0.75 to 1.92. Although no statistically significant differences were observed for dairy flavour, FST 2.11 received the highest average dairy flavour intensity score (4.00), compared with the other beverages, which scored below 2.42. The unfermented control was perceived to have significantly lower viscosity as a mouthfeel attribute than fermented beverages.

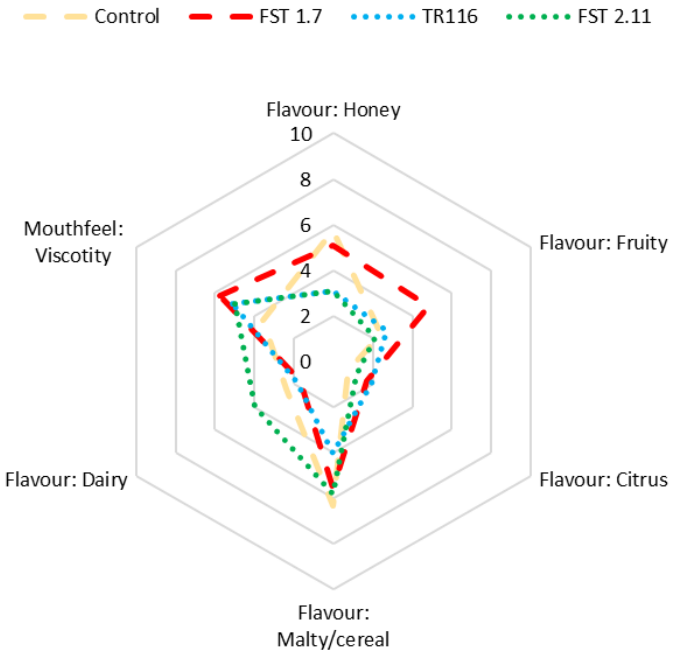


Figure 6.6: Sensory evaluation of unfermented control and fermented ELF-based beverages including *Lactiplantibacillus plantarum* FST 1.7, *Leuconostoc citreum* TR116 and *Lactobacillus amylovorus* FST 2.11.

6.4. Discussion

Incorporating side-streams into food and beverage applications is appealing from a sustainability perspective, but often requires additional processing to achieve desirable sensory and functional properties. ELF, a decolourised upcycled BSG protein isolate, light beige/sandy in colour, exhibits high solubility properties at acidic pH values, and neutral sensory properties (Ahern et al., 2025; Jaeger et al., 2024). Considering the extensive treatment this protein ingredient has already undergone (Turck et al., 2023), further processing such as fermentation with LAB could unlock valuable benefits Fermentation with LAB, including the ones used in this study (*L. plantarum* FST 1.7, *L. citreum* TR116 and *L. amylovorus* FST 2.11), have already shown to improve sensory and functional properties of brewing by-products (Jaeger et al., 2024; Neylon et al., 2021; Neylon et al., 2023; Wätjen et al., 2023). This makes ELF an interesting high-protein substrate (~87%), for developing high-protein fermented LAB beverages (Ahern et al., 2025). Therefore, this study investigated the potential of ELF as a substate for single-strain LAB fermentation and assessed the techno-functionality and sensory attributes of the final beverages.

The growth of the LABs suggests that the high protein ELF-based beverages served as a favourable substrate for all strains.TR116 demonstrated significant growth during the first 24 hrs and reached its highest cell count after 48 hrs. Neylon et al. (2023), Rice et al. (2020) and Sahin et al. (2024) reported that this strain achieved maximum cell counts after 24 hrs using brewers spent rootlets, cereal-based worts (barley, oat and wheat) as well as legume flour as substrates, before entering the stationary, and later, death phase. In sourdough production, Hoehnel et al. (2020) and Sahin et al. (2019) observed this strain reaching maximum cell count even more rapidly, after just 6 and 12 hrs growth periods, respectively. LABs including TR116 can be sensitive to environmental stressors such as osmotic pressure, pH and imbalances in essential micronutrients, all of which can slow their growth (Papadimitriou et al., 2016). The relatively high sodium content, low vitamin availability, and near alkaline pH of the ELF beverage may have contributed to a delayed growth, requiring a longer adaptation period (Ahern et al., 2025). FST 2.11 displayed slow but progressive growth, reaching only 7.7 log CFU/ml at the final time point. As a homofermentative strain, its metabolism can be primarily restricted to the fermentation of a single hexose, and this combined with environmental stressors may have contributed to its slower growth (Von Wright & Axelsson, 2019). FST 2.11 exhibited a pH reduction of 2 units to a mildly acidic level, yet it has demonstrated tolerance

at much lower pH conditions (Peyer et al., 2017). Variability in substrate adaptability suggests that extended fermentation could support continued growth of FST 2.11 beyond 48 hrs, due to its slow adaptation to the ELF beverage (Jaeger et al., 2024; Peyer et al., 2017). FST 1.7 growth in the ELF beverage aligned more consistently with previous studies, achieving a maximum cell count after 48 hrs (8.5 log CFU/ml) and a pH drop of 2 units (Neylon et al., 2023; Peyer et al., 2015). The ELF-based beverages initially exhibited a relatively high pH (~ 8) prior to fermentation, exceeding that of other brewing by-product substrates, which are generally ≤ pH 6, however, the pH decrease of 1-2 units after 48 hrs of fermentation aligns with values reported in other studies (Jaeger et al., 2024; Neylon et al., 2023). Elsewhere, protein-rich fermented beverages formulated with whey (starting pH ~6.3), casein (starting pH ~7) or a tofu byproduct (liquid fraction starting pH ~5.3) protein were found to achieve a final pH of less than 4.5 after 48 hrs or less of fermentation (Alrosan et al., 2024; Cho et al., 2015; Tu et al., 2019). Therefore, the refined, protein-dominant composition of ELF, compared to more complex substrates such as whey or cereal-based matrices, may have imposed additional metabolic stress and contributed to delayed growth and prolonged adaptation. LAB fermentation offers a biological approach for beverage acidification in the development of protein fortified soft drinks without the need of acidulants or relying on carbonation. However, pH values above 4.5 like the ones in this study should be carefully considered as they may create conditions that allow pathogen bacteria to grow in the final product, where unfermented protein soft drinks have acidic values between 2.9 to 4.2 (Ahern et al., 2023; Steen & Ashurst, 2006). Extended fermentation time, mixed fermentations with complementary LAB strains, or the addition of acidulants would be necessary in this case to produce a more acidic end product (Ashurst et al., 2017; Cho et al., 2015).

Sucrose served as the primary carbon source for supporting the growth of the LAB strains in the ELF beverage. Sucrose fermentation generally involves the transport of sucrose into the microbial cell using the permease transport system, where it is phosphorylated to sucrose-6-phosphate. Inside the cell this is then broken down enzymatically (by sucrose-6-phosphate hydrolase) into free fructose as well as glucose-6-phosphate, that can directly enter glycolysis. In addition to lactic acid, this pathway leads to the production of significant amounts of CO2 and ethanol or acetate (Von Wright & Axelsson, 2019). While this pathway applies to the heterofermentative strains FST 1.7 and TR116, FST 2.11, a homofermentative LAB strain, does not follow the same route (Cui et al., 2021). After sucrose is broken down it goes through the Embden Meyerhof Parnas pathway, whereby glucose or fructose are converted to pyruvate

and enzymatically (by lactate dehydrogenase) reduced to lactic acid (Von Wright & Axelsson, 2019). This strain exhibited the most extensive sucrose breakdown but also the highest residual glucose. All strains exclusively produced lactic acid as the main organic acid, with FST 1.7 and FST 2.11 generating similar values highlighting their strong acid producing capabilities (Peyer et al., 2017), while TR116 generated slightly lower amounts, proportional to the pH and TTA values of the strains. While FST 1.7 has been previously documented to produce, to some extent, quantities of acetic acid, TR116 generally produces high amounts of acetic acid, especially when mannitol is formed (Neylon et al., 2023; Nyhan et al., 2023). However, acetic acid levels in the TR116-fermented beverage were below the limit of quantification (≤ 0.003g/100ml), with 0.58 g/100ml mannitol produced, indicating specific fermentation substrates and conditions might minimise the acetate production.TR116 was found to excel in fructose utilisation. When free fructose is present, the enzyme mannitol-2-dehydrogenase catalyses the reduction of fructose to mannitol, simultaneously regenerating NAD+, a necessary co-factor for acetate kinase to produce acetic acid. This process links the production of mannitol and acetate during the metabolic pathway of TR116 (Martínez-Miranda et al., 2022; Rice et al., 2020; Sahin et al., 2021). This strain has already shown to be an ideal candidate in reducing sugar of different bakery products, such as burger buns, and cereal-based worts, as well as in apple juice, where a sugar reduction of 83% was achieved alongside the production of 61.6 g/L mannitol (Rice et al., 2020; Sahin et al., 2019). According to the Regulation (EC) No 1924/2006 for a beverage to carry a “low sugar” claim it must contain no more than 2.5 g/100ml of sugar, while “sugar free” claim is only allowed if the beverage contains no more than 0.5g/100ml European Parliament & of the Council. (2006). All fermented ELF beverages retained sucrose contents higher than 4g/100ml and therefore did not satisfy these claims. Beverages facilitate easier overconsumption of sugar/calories compared to solid food. Protein-fortified beverages might alleviate this by enhancing satiety and fullness (Morell & Fiszman, 2017; Solah et al., 2010). TR116 shows potential for use in sugar-reduced protein soft drinks, offering an alternative to commonly used artificial sweeteners, which have been associated in some studies with gastrointestinal problems and metabolic diseases (Ghusn et al., 2023).

Soft drinks and non-alcoholic water-based beverages are typically expected to exhibit clear to semi-transparent appearances (Steen & Ashurst, 2006). The fermented ELF-based protein beverages exhibited lower L* whiteness values compared to the unfermented control, as reflected in the RBG images. This may be attributed to the occurrence of non-enzymatic

browning (Maillard reaction) during the sterilisation step. In addition, the fermented beverages contained higher amounts of reducing sugars, whereas the control contained mostly non-reducing sucrose, therefore darkening the beverage, as well as turbidity from the fermentation process (El Hosry et al., 2025). Turbidity is inevitable in fermented beverages due to the suspended bacterial cells and particles that lead to a cloudy appearance. Beverages with this characteristic are sometimes filtered to enhance clarity, such as kombuchas or water kefirs, unlike opaque fermented beverages such as milk kefir where consumers anticipate their naturally creamy look (Coelho et al., 2020; Guzel-Seydim et al., 2021). The ELF-based beverage fermented by FST 1.7 showed the highest turbidity and the greatest separation rate, followed by FST 2.11, which exhibited notable turbidity but a lower separation rate than TR116. Despite having had the highest viable cell count, TR116 displayed the lowest turbidity and a markedly lower separation rate than FST 1.7. These turbidity differences can be caused by several factors. *Leuconostoc citreum* is a well-known homo-exopolysaccharide (EPS) producing species capable of enzymatically synthesising EPS such as dextran from sucrose (Zannini et al., 2016) EPS-producing LAB strains are known for their ability to enhance techno-functional properties, notably improving viscosity and colloidal stability of low fat, plant-based and gluten free products (Hernández-Figueroa et al., 2025). They have also been used in the production of LAB fermented plant protein-fortified beverages (Camargo Prado et al., 2015; Goveas et al., 2021). The observations in ELF-based beverages fermented by TR116 could be linked with its EPS production, increasing viscosity and contributing to the formation of a more unified colloidal system (Liu et al., 2020; Zhang et al., 2023). This observation aligns with the tribology results, where TR116-fermented beverages showed the highest lubricating properties. However, it contradicts the viscosity results whereby TR116 was the least viscous out of the fermented beverages, although these results did not different significantly between samples. Therefore, even small quantities of EPS may contribute positively to colloidal stability and lubricating properties.

Additionally, these outcomes could also be affected by the final beverage pH. Since beverages fermented by FST 1.7 or FST 2.11 dropped to lower pH values compared to TR116, closer to the isoelectric point of ELFs (~3.4), cause protein – protein complexes may have been formed in the beverage, affecting clarity and increasing instability (Ahern et al., 2025; Laclair & Etzel, 2010). Furthermore, high organic acid levels in beverages fermented by FST 2.11, combined with extensive sucrose reduction, likely contributed to the observed highest frictional behaviour (Laguna & Sarkar, 2017; Upadhyayet al., 2016). In contrast, FST 1.7-fermented

beverages, with the most residual sucrose, showed Stribeck curves similar to the unfermented control.

Fermentation can significantly alter the sensory profile of a product. FST 2.11 produced the most intense dairy flavour. Although this did not reach statistical significance, this trend aligns with previous reports indicating its ability to generate secondary metabolites such as 2, 3 butanedione, diacetyl, and acetoin which impart perceived buttery or creamy notes and contribute to the dairy like sensory profile of fermented cereal substrates like the ELF-based beverage (Blandino et al., 2003; Peyer et al., 2017). However, these dairy notes can be found to be undesirable in other products such as wines and beers, and additionally in soft drinks (Ibrahim & Ouwehand, n.d.). FST 1.7 has been previously described to produce pronounced fruity flavours. For instance, both Nyhan et al. (2023) and Neylon et al. (2023) demonstrated that non-alcoholic sour beers and spent rootlets in a bread system, respectively, showed higher fruity notes compared to other LAB strains, attributed to significantly high ester production as well as reduced diacetyl levels. This supports the sensory findings in the current study, where the FST 1.7-fermented ELF-beverage was perceived by the panellists as having significantly higher fruity flavours compared to other LAB, suggesting that fruity flavour production is a common trait of FST 1.7 across diverse cereal-based matrices. However, this is not always the case. Peyer et al. (2015) showed that fermentation of wort using FST 1.7 produced elevated amounts of diacetyl and acetoin demonstrating these strains can also take alternative routes in their metabolic pathway. The TR116-fermented ELF-beverage exhibited significantly reduced malty/cereal and honey flavours. All strains produced insufficient amounts of organic acids for the panellists to perceive any sour taste. Additionally, the panel reported a significant increase in viscosity-based mouthfeel from unfermented to fermented samples but were unable to distinguish differences between strains. In contrast, tribological measurements revealed strain-dependent differences in lubrication, which are often correlated with sensory perception, suggesting the panellists were either not sensitive enough to detect these differences or that the tribo-cell is more sensitive than human perception, highlighting differences that the panel could not perceive (Selway & Stokes, 2013).

Typically, LAB-fermented beverages are refrigerated after fermentation in order to preserve the beneficial live probiotic bacteria, which is desired by some consumers. In this study, the product was pasteurised post-fermentation to maintain a microbially safe product, as the primary focus was on sensory and techno-functional properties rather than probiotics effects. Non-viable LAB and their fermentation-derived metabolic by-products, known as postbiotics,

have shown emerging evidence of conferring health benefits. Therefore, while pasteurised, these beverages might show presence of compounds such as short chain fatty acids (SCFAs), EPS, organic acids, peptides, enzymes and cellular fragments, potentially contributing to the beverages' health related functional properties (Chang et al., 2021; Żółkiewicz et al., 2020). However, further research is required to investigate this in more detail.

These results demonstrate the suitability of upcycled ELF as a substrate for LAB fermentation producing a promising base for a high protein fermented plant protein beverage. Further formulation such as the incorporation of carbonation, acidulants or flavouring agents, may further enhance the development of these novel high-protein beverages.

6.5. Conclusion

This study demonstrates that brewing side-streams, such as ELF, can be formulated into high protein fermented beverages. The three LAB strains exhibited distinct fermentation behaviours, with TR116 supporting the most rapid microbial growth and mannitol formation, while FST 1.7 and FST 2.11 showed the greatest acidification and therefore highest reduction in pH. These strain specific metabolic activities contributed to measurable differences in viscosity, turbidity, separation rate and lubrication characteristics, indicating that fermentation can tailor the techno-functional attributes of beverages to suit different product profiles. Sensory attributes compared to the unfermented control were improved, highlighting the potential of the ELF-based beverage as a viable substate. This base shows potential for developing novel beverages using sustainably sourced protein, expanding opportunities in fortified protein soft drinks and non-alcoholic drinks beyond milk alternatives. Future studies are required to optimise shelf life and flavour as well as exploring the potential effects of co-culutre LAB fermentations to further enhance product performance and consumer acceptance.

6.6. Acknowledgement

The authors would like to thank particularly Celia Segura Godoy for her contributions to the sugars and organic acids of the samples as well as her invaluable expert advice and insight. The authors would also like to thank Dr Michael O Grady and Dr Karen Galvin for their technical support, and EverGrain LLC for kindly providing EverPro™ Light Fraction (ELF).

6.7. Supplementary Material

Supplementary Table 6.1: Attributes of the sensory analysis and their descriptors.

Attribute	Description
Taste: Sweet	Perceived sweetness
Taste: Sour	Perceived sourness/acidity
Flavour: Honey	Perceived sweetness
Flavour: Fruity	Perceived fruit flavour (peach like flavour)
Flavour: Citrus	Zesty and tangy sharp notes (lemon like flavour)
Flavour: Earthy/Muddy	Flavour associated with earth/dirt
Flavour: Malty/Cereal	Perceived malt/cereal flavour
Flavour: Dairy	Flavour associated with dairy products (cream/milk)
Mouthfeel: Viscosity	Thickness/richness of beverage

Supplementary Table 6.2: pH, TTA and bacterial cell count results at different fermentation times (0, 24 and 48hrs) of unfermented control and fermented ELF-based beverages including *Lactiplantibacillus plantarum* FST 1.7, *Leuconostoc citreum* TR116 and *Lactobacillus amylovorus* FST 2.11. Data are expressed as mean ± standard deviation**.** Values in the same row that share a letter do not differ significantly ($p< 0.05$). n.a = not applicable.

	Control	FST 1.7	TR116	FST 2.11
pH (T0)	8.02 ± 0.06	7.93 ± 0.03 [a]	7.93 ± 0.05 [a]	7.97 ± 0.02 [a]
pH (T24)	n.a	7.53 ± 0.07 [a]	6.99 ± 0.06 [b]	7.37 ± 0.08 [c]
pH (T48)	n.a	5.42 ± 0.22 [a]	5.88 ± 0.05 [b]	5.47 ± 0.16 [a]
Cell count T0h (Log CFU/ml)	n.a	6.99 ± 0.09 [a]	6.93 ± 0.08 [a]	6.75 ± 0.08 [b]
Cell count T24h (Log CFU/ml)	n.a	7.70 ± 0.11 [a]	8.68 ± 0.39 [b]	7.23 ± 0.11 [c]
Cell count T48h (Log CFU/ml)	n.a	8.53 ± 0.43 [a]	9.21 ± 0.18 [b]	7.66 ± 0.10 [c]

Supplementary Table 6.3: Quantification of sugar and acids at times 0, 24 and 48 hrs expressed in g/100ml of unfermented control and fermented ELF-based beverages including *Lactiplantibacillus plantarum* FST 1.7, *Leuconostoc citreum* TR116 and *Lactobacillus amylovorus* FST 2.11. LOQ is defined as below limit of detection i.e levels below 0.003g/100ml acids, 0.005g/100ml sugars. Data are expressed as mean ± standard deviation**.** Values in the same column that share a letter do not differ significantly ($p< 0.05$).

	Lactic acid	**Acetic acid**	**Sucrose**	**Glucose**	**Fructose**	**Mannitol**
Control	0.00 ± 0.00 ^{a}	0.00 ± 0.00	5.36 ± 0.21 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
FST 1.7 (T0)	0.00 ± 0.00 ^{a}	0.00 ± 0.00	5.12 ± 0.19 ^{ab}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
FST 1.7 (T24)	0.03 ± 0.00 ^{ab}	0.00 ± 0.00	4.98 ± 0.16 ^{abc}	0.01 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
FST 1.7 (T48)	**0.31 ± 0.04** ^{d}	**0.00 ± 0.00**	**4.55 ± 0.33** ^{cd}	**0.05 ± 0.01** ^{a}	**0.07 ± 0.02** ^{bc}	**0.00 ± 0.00** ^{a}
TR116 (T0)	0.00 ± 0.00 ^{a}	0.00 ± 0.00	5.37 ± 0.14 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
TR116 (T24)	0.05 ± 0.00 ^{b}	≤ LOQ	5.04 ± 0.09 ^{abc}	0.01 ± 0.00 ^{a}	0.04 ± 0.00 ^{b}	0.04 ± 0.00 ^{b}
TR116 (T48)	**0.14 ± 0.01** ^{c}	**≤ LOQ**	**4.36 ± 0.14** ^{d}	**0.01 ± 0.00** ^{a}	**0.17 ± 0.02** ^{d}	**0.58 ± 0.04** ^{c}
FST 2.11 (T0)	0.00 ± 0.00 ^{a}	0.00 ± 0.00	5.05 ± 0.11 ^{ab}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
FST 2.11 (T24)	0.04 ± 0.01 ^{ab}	0.00 ± 0.00	4.75 ± 0.12 ^{bcd}	0.21 ± 0.06 ^{a}	0.09 ± 0.01 ^{c}	0.00 ± 0.00 ^{a}
FST 2.11 (T48)	**0.31 ± 0.06** ^{d}	**0.00 ± 0.00**	**2.87 ± 0.51** ^{e}	**1.15 ± 0.34** ^{b}	**0.14 ± 0.04** ^{d}	**0.00 ± 0.00** ^{a}

Supplementary Table 6.4: Friction factors of unfermented control and fermented ELF-based beverages including *Lactiplantibacillus plantarum* FST 1.7, *Leuconostoc citreum* TR116 and *Lactobacillus amylovorus* FST 2.11 at different sliding speeds. Data are expressed as mean ± standard deviation. Values in the same row that share a letter do not differ significantly ($p< 0.05$).

	Control	**FST 1.7**	**TR116**	**FST 2.11**
Sliding speed (m/s)		**Frictional factor**		
10^{-6}	0.063 ± 0.001 ^{ab}	0.053 ± 0.011 ^{b}	0.030 ± 0.005 ^{c}	0.068 ± 0.006 ^{a}
10^{-4}	0.204 ± 0.041 ^{a}	0.188 ± 0.036 ^{a}	0.091 ± 0.013 ^{b}	0.238 ± 0.030 ^{a}
10^{-2}	0.083 ± 0.010 ^{bc}	0.101 ± 0.009 ^{b}	0.065 ± 0.015 ^{c}	0.141 ± 0.019 ^{a}
1	0.013 ± 0.002 ^{a}	0.011 ± 0.001 ^{a}	0.011 ± 0.001 ^{a}	0.12 ± 0.001 ^{a}

Supplementary Table 6.5: Results of the sensory analysis of unfermented control and fermented ELF-based beverages including *Lactiplantibacillus plantarum* FST 1.7, *Leuconostoc citreum* TR116 and *Lactobacillus amylovorus* FST 2.11. Data are expressed as mean ± standard deviation. Values in the same row that share a letter do not differ significantly ($p< 0.05$).

	Control	**FST 1.7**	**TR116**	**FST 2.11**
Taste: Sour	4.58 ± 2.15 ^{a}	4.92 ± 1.73 ^{a}	4.25 ± 1.82 ^{a}	3.83 ± 2.25 ^{a}
Taste: Sweet	1.08 ± 1.51 ^{a}	2.25 ± 1.76 ^{a}	1.92 ± 1.51 ^{a}	2.17 ± 1.8 ^{a}
Flavour: Honey	5.75 ± 2.56 ^{a}	5.08 ± 2.27 ^{ab}	3.08 ± 1.51 ^{b}	3.08 ± 1.93 ^{b}
Flavour: Fruity	2.42 ± 1.24 ^{a}	4.83 ± 1.64 ^{b}	2.67 ± 1.15 ^{a}	2.08 ± 1.38 ^{a}
Flavour: Citrus	0.75 ± 1.14 ^{a}	1.67 ± 1.56 ^{a}	1.92 ± 1.00 ^{a}	1.17 ± 1.27 ^{a}
Flavour: Malty/cereal	6.33 ± 1.87 ^{a}	5.58 ± 1.31 ^{ab}	4.00 ± 1.6 ^{b}	5.83 ± 1.27 ^{a}
Flavour: Dairy	2.42 ± 2.23 ^{a}	1.67 ± 1.92 ^{a}	1.83 ± 2.21 ^{a}	4.00 ± 2.52 ^{a}
Mouthfeel: Viscosity	3.58 ± 1.44 ^{a}	5.75 ± 1.36 ^{b}	5.08 ± 1.38 ^{b}	5.00 ± 0.95 ^{b}

7. Chapter 7

7.1. Discussion, conclusions, and future work.

This thesis set out to evaluate the potential of protein from brewers spent grain, BRPI, as a sustainable, functional and nutritionally valuable ingredient for application in acidic, water-based beverage systems. This objective was achieved through market analysis, an *in vitro* digestion study, techno-functional characterisation and application in trial beverage systems. Collectively, the findings demonstrate that both BRPIs can address several key limitations currently restricting the use of PBPs in soft drinks, including poor solubility, limited digestibility and unfavourable sensory attributes. Importantly, this work demonstrates feasibility of incorporating upcycled protein ingredients into commercial relevant products.

The current protein soft drink market, Chapter **2**, highlights a clear imbalance between consumer demand for convenient high protein RTD beverages and the limited use of PBPs. Animal based proteins, particularly whey, dominate the market attributed to its excellent solubility in acidic conditions and high protein quality (FAO, 2013; Goulding et al., 2019; Herreman et al., 2020; Tang et al., 2023), alongside collagen, despite its poor EAA profile (Gauza-Włodarczyk et al., 2017; Phillips, 2017). Their use conflicts with increasing demand for sustainable and plant-based alternatives, with the PBP beverages limited by low protein contents and reliance on free amino acid fortification to compensate for technical limitations in acidic water-based systems. Importantly, this market analysis establishes key functional and nutritional benchmarks required for successful formulation and commercialisation including high solubility at low pH and competitive protein quality. This highlighted a clear opportunity in the current protein soft drink market for alternative PBP sources, like BRPI, that match the techno functional properties of ABP.

A key challenge within the current market is the inability of PBPs to deliver high solubility under acidic conditions. Chapter **3** firstly provides the techno functional characterisation of commercial WPI and PBPs such as pea, soy, rice and barley rice protein, for their potential suitability for specific applications. Comparable to WPI, the findings demonstrate that BRPI addresses this challenge, exhibiting a high solubility at low pH and high protein content. This makes it favourable for application in acidic beverage systems like soft drinks addressing the core technical barrier for PBPs seen in Chapter **2**. The determination of novel nitrogen to

protein conversion factors for all the protein isolates, specifically for BRPI, provides essential compositional data facilitating its use for future studies. The dynamic *in vitro* digestibility results expanded understanding of PBP bio-accessibility and DIAAS. BRPI exhibited competitive nitrogen bio accessibility (90%) and DIAAS (67%), against the other PBP studied. However, being limited in the EAA lysine, animal proteins like WPI, maintain superior nutritional quality. Even so, the combined functional and nutritional performance of BRPI bridges the gap between plant and animal proteins in acidic beverage formulation.

Despite the functional advantages of BRPI, colour is a critical attribute of protein ingredients with a colour closer to white having wider applicability. Chapter **4** addresses sensory as well as visual limitations of the dark brown colour of EDF, by a decolourised form, ELF, presenting a lighter beige/sandy colour. This work demonstrated that targeted processes like decolourisation, can effectively overcome these barriers. The resulting ingredient, ELF exhibited improved solubility, reduced undesirable flavour attributes and enhanced mouthfeel, while retaining a high protein content (>80%) and overall functionality (Sakai et al., 2022). The findings highlight the critical role of processing in unlocking the application potential of PBPs, enabling their used in a wider range of applications and flavour profiles. Although, EU regulatory restrictions limits ELFs commercialisation, indicating a trade-off between visual appeal, functionality and regulatory feasibility (European Parliament & Council, 2008). However, EDF supports applications for darker or strongly flavoured products such as in cola, chocolate or coffee, demonstrating flexibility in ingredient use depending on product context.

With BRPI exhibiting favourable digestibility characterises and high solubility at low pH, these ingredients were identified as promising candidates for high protein acidic beverage applications. Chapter **5** features the incorporation of BRPI in a range of commonly consumed soft drinks and non-alcoholic beverages demonstrating its practical feasibility. The findings demonstrated BRPI can achieve commercial level protein fortification at high protein contents (8%) across diverse acidic beverage systems addressing the limitations of PBPs seen in Chapters **2**. Protein concentration was identified as a key driver of system behaviour, influencing pH, aggregation and stability notably near the isoelectric point (Laclair & Etzel, 2010). Critically, higher levels improved beverage clarity and stability, indicating that formulation strategies can be optimised to enhance beverage performance. These findings show BRPI as a suitable candidate for development of PBP soft drinks suitable for individuals who do not meet their daily protein requirements.

Beyond its direct application as a functional ingredient, Chapter **6,** explored the potential of BRPI, as a substate for LAB fermentation to produce a high protein fermented beverage. BRPI proved to be a suitable substrate for the three different LAB. All strains produced organic acids highlighting an opportunity for natural acidification suggesting the possibility of reducing the need for added acidulants in soft drinks. Techno-functionality outcomes were strain specific, while *Leuconostoc citreum* TR116 producing mannitol, offering potential as a sugar replacement for low sugar or sugar reduced soft drinks (Rice et al., 2020). Each strain also developed a distinct flavour profile revealed by the sensory panel. *Lactobacillus amylovorus* FST 2.11, produced dairy notes, unsuitable for soft drinks applications, whereas *Lactiplantibacillus plantarum* FST 1.7 produced fruity notes making it more appropriate (Nyhan et al., 2023). This indicates fermentation can serve as multifunctional tool to simultaneously improve flavour, reduce sugar and enhance functional profile including natural acidification and potentially carbonation of these types of beverages. Moreover, the natural turbidity of fermented beverages may help address clarify issues associated with protein incorporation, aligning with consumer expectations.

7.1.1. Conclusion

This thesis demonstrates the significant potential of the underutilised side stream, BRPI as a sustainable, functional and nutritional valuable protein source for acidic beverage applications. Its favourable solubility in acidic conditions, digestibility and functionality across diverse beverage systems highlights its applicability as a novel ingredient, to address the market gap for PBP soft drinks and non-alcoholic beverages that shifts away from heavy milk-based alternatives to hydrating water-based drinks. Targeted modification strategies such as decolourisation and fermentation are essential for overcoming sensory and functional limitations. Moreover, LAB fermentation introduces new developments for natural acidification and flavour enhancement with sugar reduction in protein soft drinks. These finding contribute and support sustainable novel protein beverage products that support the use for consumers with inadequate protein intake like athletes, individuals on plant-based diets and GLP-1 therapy users.

7.1.2. Future work

Building on the finding of this thesis, future research should focus on the optimisation of BRPI based beverages to enhance their functionality, sensory appeal and consumer viability. Beyond beverage applications, the combination of having a high protein content and oil holding

capacity suggests BRPI could also be explored in other food systems, including meat analogues and high protein bakery products like crackers and biscuits. Given the decolourisation process currently used to produce ELF restricts its commercialisation in the EU. Alternative approaches, such as enzymatic treatments or heat treatments need to be looked into, while preserving nutritional and functional quality. Long term studies assessing shelf life, microbial safety and storage stability are essential aspects that needs to be considered for product quality. Further formulation work is required, including the impact of added acidulants to achieve higher protein concentrations while maintaining acidity levels expected of these types of beverages that also impact flavour and shelf life. Exploring co-culture LAB fermentation or SCOBY could expand the range of their flavour profiles and improve acidification for fermented high protein beverages. Investigating the health aspects of these final products is also of interest, such as digestibility that can be affected by the food and beverage matrix of the final product and in terms of fermented product the postbiotic components. Incorporating two or more PBPs to achieve a more complete essential amino acid profile should also be considered, as BRPI is limited in the essential amino acid lysine. Therefore, the inclusion of a complementary protein, such as a legume protein (pea, chickpea, lentil, or faba bean) would be appropriate following functionality analysis as well as digestibility. Sensory analysis is then needed to ensure consumer acceptance of the final products but also the use of advanced sensory and tribological techniques could be critical for the development of these products that align with consumer expectations. Optimising carbonation processes will also be critical for developing carbonated protein soft drink or non-alcoholic beverage, particularly to manage foaming issues expected from the protein inclusions. Finally, a life cycle assessment would provide valuable information on the real environmental impact of these sustainable protein products.

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Oral presentations

2022: UCC/IFSTI 50th Food science and Technology Conference. UCC, Cork.

2023: 20th European Young Cereal Scientists and Technologists Workshop. Leuven, Belgium.

2023 & 2024: Annual meeting with Anheuser InBev Busch. UCC, Cork.

Poster presentations

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